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Development of microwave-assisted methods to aid in carbohydrate analysis

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DEVELOPMENT OF MICROWAVE-ASSISTED METHODS TO AID IN CARBOHYDRATE ANALYSIS

BY

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TBALE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

ABSTRACT

DEVELOPMENT OF MICROWAVE-ASSISTED
METHODS TO AID IN CARBOHYDRATE ANALYSIS

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This study involved development of three high-throughput chemical techniques to assist in carbohydrate analysis. The methods utilized both microwave radiation and a volatile organic base to achieve short reaction times and high product yield. Two methods, one reductive and one non-reductive, released intact O-glycans for structural analysis, and one method aided in determination of the site of O-glycosylation on the peptide.

Optimal reaction conditions for each method were determined on standard glycoproteins. In comparison to common release methods, microwave-assisted reductive O-glycan release provided higher yields of O-glycans in every case. O-glycans were released rapidly and were able to be subsequently labeled for chromatographic separations by the non-reductive release method. The O-glycosylation site was determined following release by the formation of peptide-dimethylamine adducts, which, coupled with the addition of a deuterium label further aided in selection of glycopeptides for analysis.

CHAPTER 1

INTRODUCTION

Carbohydrates are the most abundant group of naturally occurring biomolecules. The majority of eukaryotic cell-surface and secreted proteins are glycosylated.[1] Glycosylation is a post-translational modification that imparts a variety of biological roles including structure and stability, recognition, adhesion and immune response.[2,3]

Glycan Structural Features

Structurally, the carbohydrate is typically bound to a protein through the nitrogen of asparagine (Asn), forming N-glycans, or through the oxygen of serine (Ser) or threonine (Thr), forming O-glycans. N-glycans share a common pentasaccharide core; with two N-acetylglucosamine and three mannose residues (protein-$GlcNAc_2Man_3$). Extensions from the mannose residues of the N-glycan core are called antenna and form three major classes: high mannose, complex, and hybrid type, which are shown in Figure 1.1. The most abundant O-glycans are mucin type which are bound to the protein via N-acetylgalactosamine (GalNAc). Conversely, O-glycans do not contain one common core structure,

eight mucin-type core structures have been reported and are shown in Figure 1.1, core 1-4 are the most commonly observed.

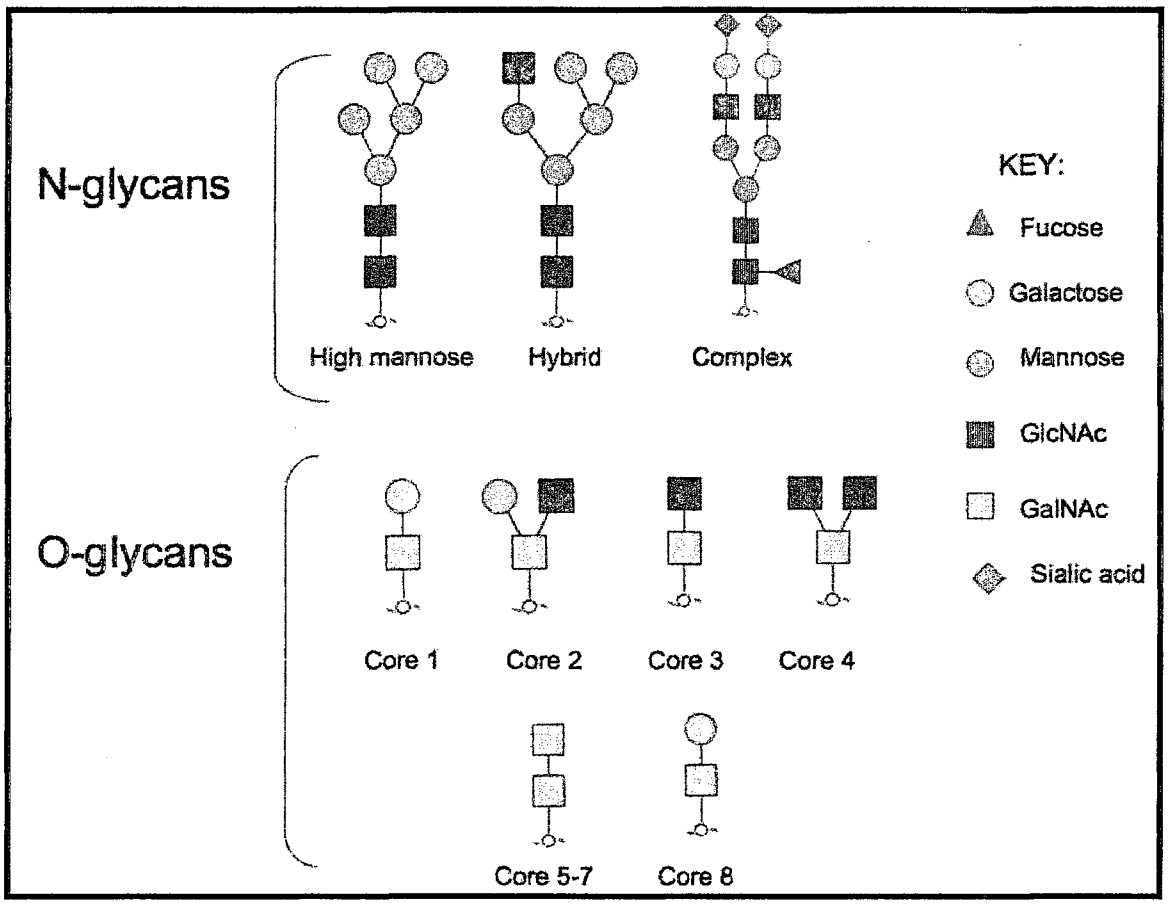


Figure 1.1: N and O-glycan core structures (linkage positions not shown)

Mucin type O-glycans are commonly found in secretions and as membrane bound glycoproteins, they form mucosal linings and contribute to the hydrophilicity of mucins in mucosal linings. This type of O-glycosylation is the focus of this report. Other, less common, O-linked glycans exist such as O-glucosylation, O-fucosylation, and O-mannosylation. These O-glycosylations may function in the early stages of development, and have been found to be

associated with clotting proteins, as well as in the nervous system.[3] O-glycosylation through GlcNAc is another common O-glycosylation which occurs on numerous nuclear proteins. Many proteins which are O-GlcNAc glycosylated also exist in phosphorylated forms, and it has been suggested that O-GlcNAc and O-phosphorylation play reciprocal roles in regulation. For example, the carboxy terminal domain of RNA-polymerase II takes two forms, phosphorylated or glycosylated. The glycosylated form has been shown to initiate transcription and then is de-glycosylated and phosphorylated for elongation.[4] For O-GlcNAc type glycosylation, the site is the primary structural component because no elongation of the oligosaccharide occurs.

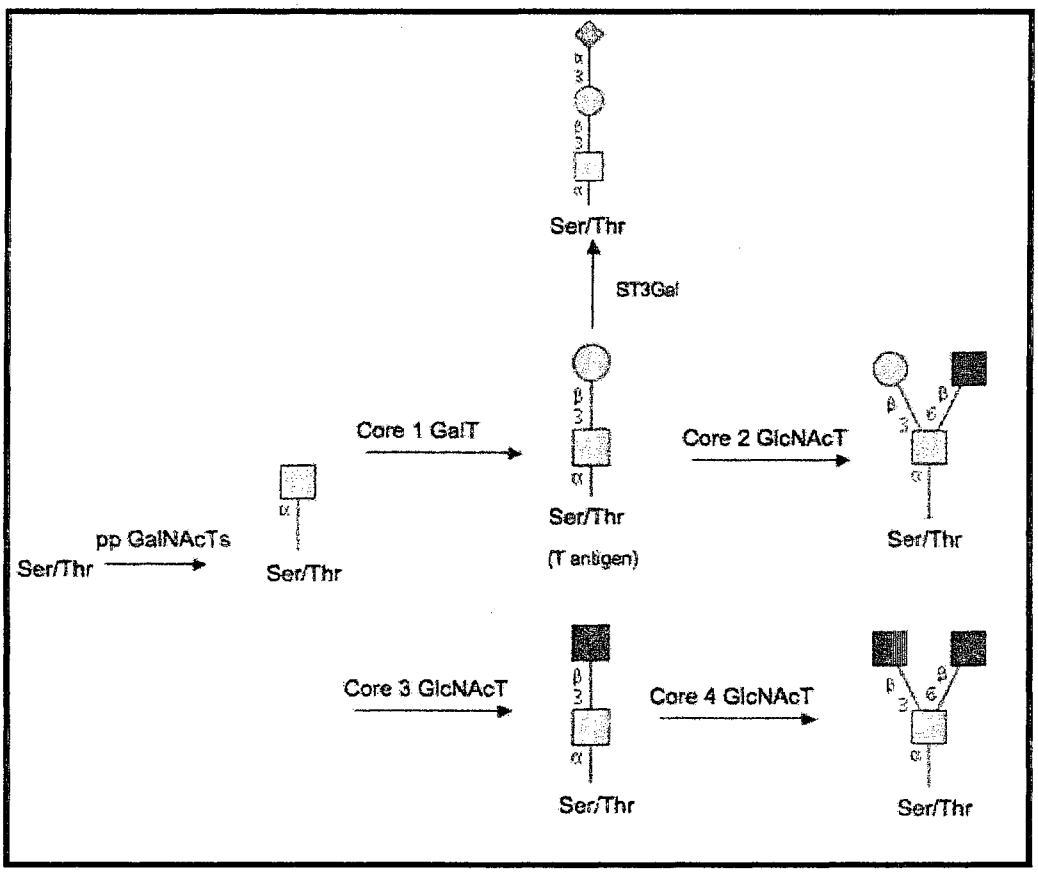
Glycoprotein Biosynthetic Pathway

Unlike proteins, wherein DNA forms a template for the amino acid sequence, elongation of the oligosaccharide chain is not a template-driven gene-encoded processes. Glycosylation is, however, dependent on enzymes available and local cell environment. N-glycosylation is initiated in the endoplasmic reticulum (ER) by the addition of a pre-assembled lipid-linked oligosaccharide precursor to an asparagine residue on the protein, this portion of the biosynthetic pathway is highly conserved. The N-glycan is further processed throughout the ER and golgi apparatus, it is in the late golgi where the structural differentiation occurs.

Mucin type O-glycosylation is normally initiated in the ER or golgi apparatus by a family of up to 15 polypeptide GalNAc transferases (ppGalNAcTs)

which transfer GalNAc to a serine or threonine residue on the protein.[5] There is evidence that O-glycosylation is initiated in the golgi,[6] however its specific location is still a matter of debate. Also, the multiple GalNAc transferases that have been reported have both different and overlapping specificities for amino acid sequences.[7] The O-glycan chain is further elongated in a stepwise manner by enzymatic transfer of monosaccharides. O-glycans which remain as O-GalNAc or are not further modified beyond the core 1 structure are known as Tn- and T-antigens respectively, and are commonly associated with tumors. The biosynthetic transferases involved in production of the four common core structures are shown in Scheme 1.1.

O-glycans are usually found in regions of the protein rich in serine, threonine, and proline residues, often in tandem repeats, but no specific amino acid consensus sequence is known at this time. Abnormal or altered glycosylation may be associated with disease states and therefore investigations of glycans and glycoproteins are potential avenues for disease diagnosis and biomarker discovery.



Scheme 1.1: Synthetic pathways of the 4 common core mucin type O-glycans. The core structures are further elongated by specific transferases (T) such as the addition of sialic acid to core 1.

Carbohydrates as potential biomarkers have been investigated since the 1970s in which differences in glycosylation between patients with cancer and healthy controls were observed through lectin binding.[8] To date, detailed structural analysis of carbohydrate chains involved in cancer and other diseases is an ongoing effort.

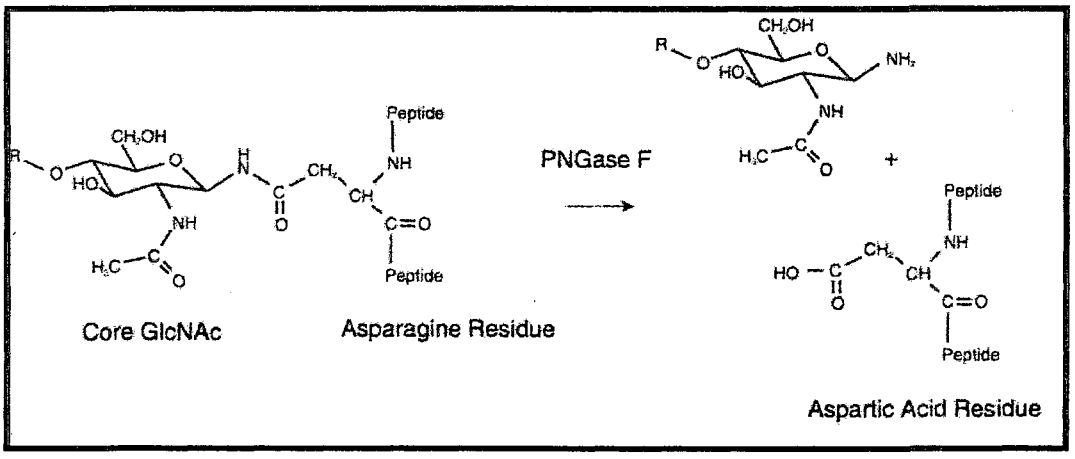
A well studied example of aberrant O-glycosylation with a connection to disease state is human immunogloublin A1 (IgA1). IgA1 is a serum protein containing a 17 amino acid hinge region with multiple serine and threonine

residues. It has been shown that four mucin-type O-glycans exist in healthy patients. Patients who suffer from IgA nephropathy (IgAn), an autoimmune disorder leading to irreversible kidney failure in 20-40% of patients, have been shown to have enriched levels of the Tn O-glycan. Through binding studies involving lectins that recognize terminal GalNAc, a qualitative increase in binding of IgAn has been observed consistently in comparison to healthy controls. This information indicates differential glycosylation patterns in IgAn patients and healthy patients.[9] It is of particular interest to determine if disease patients have different O-glycan structures than healthy patients and if specific sites of aberrant glycosylation can be found in order to target these different biosynthetic pathways in treatment.

De-glycosylation strategies

A complete structural analysis of a biological glycan includes determination of monosaccharide composition, topology, linkage analysis and the linkage site on the protein. Although there are only 9 common monosaccharides found in humans, they can be assembled into more than 15 million simple tetrasaccharides.[10] Each monosaccharide in the oligosaccharide has 3 or 4 potential attachment sites for linkages, which allows for branching. Additionally, each glycosidic linkage may be in an α or β configuration. Due to this vast potential for isomers and microheterogeneity, the glycan must first be released and separated from the protein prior to structural analysis.

Release of N-glycans is typically carried out enzymatically. Peptide N-glycosidase F, (PNGase F) is the most common endoglycosidase for universal release of N-linked carbohydrates. This amidase is able to hydrolyze the GlcNAc-Asn bond of high mannose, hybrid, and complex glycoproteins except those that contain a core 1,3 linked fucose.[11] Free native N-glycans are obtained and the protein is preserved. The former N-glycosylation (Asn) residue is converted into an aspartic acid residue as shown in Scheme 1.2. The N-glycosylation site may be determined by an isotope-coded glycosylation-site-specific tagging method utilizing ^{18}O water to introduce a mass shift in the aspartic acid.[12] A consensus sequence for N-glycosylation of: Asn-X-Ser/Thr, where X is any amino acid except proline, allows for potential glycosylation sites to be predicted in a given amino acid sequence.



Scheme 1.2: PNGase F release of N-linked glycans and conversion of asparagine to aspartic acid

O-glycan release and determination of O-glycosylation sites is challenging due to the absence of a known consensus sequence for O-glycosylation as there

is for N-glycosylation, furthermore, no known enzyme for complete de-O-glycosylation exists at this time. Specific exoglycosidases may be used to trim down the structure of core 1 O-glycans until only the core Gal-GalNAc remains which may be cleaved by O-glycosidase, an enzyme which does not affect the peptide backbone. Examples of such exoglycosidases include neuraminidase which cleaves sialic acid residues, and N-acetylglucosaminidase which cleaves the GalNAc-GlcNAc bond.[13] However, the inherent substrate specificity of the enzymes is non-ideal for use with unknown and complex samples. These enzymes are useful in determining biological function. For example, introduction of the enzyme after protein synthesis allows for morphological or physiological differences in glycosylation states to be observed and for inferences on glycan function to be made.

Since knowledge of the O-glycan structure is necessary for successful enzymatic release, non-selective chemical methods have been developed. These β-elimination based chemical methods may be divided into two general categories according to the nature of the released O-glycan: non reductive strategies and reductive strategies. Non-reductive release (further discussed in Chapter II) leaves the reducing end of the glycan free for subsequent labeling, and reductive release (further discussed in Chapter IV) reduces the reducing end GalNAc to the corresponding alditol to prevent side reactions and to aide in topology determination. A comparison of the structures of a reduced and non-reduced sugar is shown in Figure 1.2.

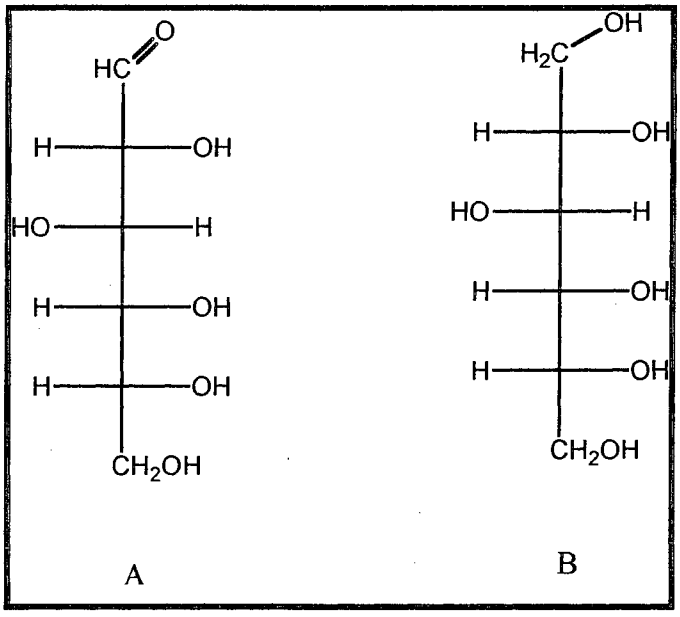


Figure 1.2: Glucose in its (A) non-reduced and (B) reduced forms.

The most common non-reductive method, release by anhydrous hydrazine, involves a six hour reaction time with extensive drying (24 hr) before the reaction to help minimize glycan degradation.[14] The most common reductive method, release with NaOH/ $NaBH_4$, is usually performed at 50°C for 16 hrs. It was the goal of this work to introduce new methods for rapid and efficient O-glycan release by utilizing the benefits of microwave radiation.

Microwave Radiation in the Chemical Field

Microwave radiation has been utilized in the chemical field since the late 1970s, with the first introduction of a laboratory microwave in 1978 by CEM Corp. Since then, the need for fast reactions and analysis as well as the push towards green chemistry have increased interest in microwave chemistry. Originally domestic microwaves (introduced in the 1940s) were utilized, however, the

inability to precisely control temperature and pressure as well as formations of hot spots lead to poor reproducibility and safety concerns.[15]

The first reported laboratory instrument that utilized microwaves was developed to analyze moisture content. Soon after, microwaves became widespread for analytical purposes such as digestions, drying and extractions, and in 1986 the first report on utilizing microwaves for chemical synthesis was published. The first microwave reactor, similar in design as the microwave used in this work, designed for chemical synthesis with temperature, pressure and safety controls, operating at 2.45 GHz was introduced in 2000.[16]

Reactions performed with microwave radiation take significantly less time relative to heating blocks or water baths to reach the reaction temperature. The heating mechanism involved is a direct transfer of energy to the reaction mixture, which is in contrast to conductive energy transfer involved with heating blocks wherein first the heating block is warmed, which then warms the reaction vessel and eventually warms the reaction mixture. Additionally, radiative heating heats all components of the sample at the same rate, instead of the contents nearest the container walls heating first. In numerous instances, it has been shown that reactions performed via microwave heating provide higher yields and cleaner reaction products in significantly shorter times compared to classical heating methods, thus their widespread use in the synthetic chemistry field.[17]

Since microwaves do not contribute to bond breaking, as the typical microwave photon has an energy of 0.037 kcal/mol, compared to the energy required to break a molecular bond of 80-120 kcal/mol, experiments were

designed by Sandoval and coworkers to determine if the so called 'microwave advantage' is solely due to shortened time to reach reaction temperature or if other factors existed. Their experiments of a PNGase F de-N-glycosylation of ribonuclease B were designed to preheat all solvents, reagents, and glassware to the reaction temperature prior to the start of the reaction. The results indicated that in some cases preheating decreased the water-bath based de-glycosylation time slightly, however, the preheating did not provide the drastically different reaction times associated with microwave heating, and it appeared that factors other than simply faster heating of the bulk solution were in place.[18]

Microwaves are able to effectively heat polar solutions due to the dipole rotation mechanism. The introduction of an electric field causes molecules to attempt to align their dipoles with the field, since the electric field is constantly oscillating, the molecules are in constant rotation. As molecules rotate, thermal energy is produced. In the biological field, water is typically used as a solvent, however, solvent alone is not the only molecule in the reaction mixture which is capable of being effected by microwaves, the starting material, catalyst or other reaction component may also be effected by microwave radiation. [19]

Since the electric field changes very rapidly, molecules do not have time to relax during irradiation, and therefore localized superheating may occur wherein much greater temperatures than the overall bulk temperature may be reached. It is this localized superheating which is believed to be the major contribution to enhanced reaction rates and yields, however, it is impossible to measure the temperature of the localized superheating.[19]

Microwave-Assisted Proteomics and Glycomics

Microwave-assisted protocols have been developed for a range of glycomic and proteomic applications. Proteolysis, often by trypsin, is a necessary step in protein characterization. This enzyme cleaves at the carboxyl-terminus of lysine and arginine residues, producing small peptides, which facilitates sequencing. Protein digestion with trypsin often includes an overnight incubation however, with use of microwave radiation, methods for rapid solution and in-gel tryptic digestions have been developed.[20,21] Quantitative protein digestions have been reported under microwave conditions of 20 min 50°C, where the digestion efficiency was 20% greater than traditional heating methods.[22] These digestion methods have been further extended by the use of trypsin immobilized magnetic beads, which produced results equivalent to or better than conventional digestion methods in as few as 15 s. The immobilized enzymatic method provides further advantages including cleaner products, since the enzyme does not contaminate the peptide mixture.[23] Pramanik *et al.* have applied microwave radiation to the tryptic digest of the tightly folded bovine ubiquitin protein which was resistant to digestion under traditional methods.[24]

Another method for protein digestion is acid hydrolysis. One example is hydrolysis with a 2% formic acid solution, which has been shown to cleave at the C-terminus of aspartic acid residues. Experiments by Hua and coworkers on the digestion of myoglobin by classical and microwave heating methods showed that

microwave heating for only 30 s provided increased peptide yield over an 8 hr heating block incubation.[25]

Another use for microwaves in the proteomic field is peptide synthesis. Peptide synthesis by chemical methods is preferred over recombinant genetics as the chemical methods allow for specific control over backbone and side chain modifications and are free from biological impurities. Chemical peptide synthesis is a time consuming process which often results in low reaction yield due to incomplete reactions.[26] One example is in the synthesis of glycopeptides for use in methodology studies and the building of reference libraries, the addition of a heavily glycosylated amino acid residue to the synthesized peptide is a very slow reaction due to steric hinderance. However, with utilization of microwave energy, the synthesis of the heavily glycosylated MUC1 peptide was accomplished in 7 hrs, comparable synthesis by traditional methods was only seen after 98 hrs.[27]

The enzymatic PNGase F N-glycan release has also been accelerated to achieve complete de-N-glycosylation in 10-60 min under microwave radiation on samples which took 24 hrs heating in a water bath.[18] However, the application of microwave radiation to de-O-glycosylation has not yet been described.

Overview of Thesis Topics

The overall goal of this work was to develop new microwave-assisted laboratory protocols for O-glycomics which fit into an overall complete sequential method for protein, N-glycan, O-glycan and glycosylation site analysis. Chapter II will discuss non-reductive strategies in the development of a microwave-assisted

non-reductive O-glycan release method for the purpose of O-glycan analysis and labeling. Chapter III will present a microwave-assisted method for complete de-O-glycosylation, peptide labeling and glycosylation site determination. Chapter IV will discuss reductive strategies and present a microwave-assisted reductive O-glycan release strategy. Finally, chapter V presents the scheme for microwave-assisted sequential N- and O- glycan release utilizing the methods developed in the previous chapters.

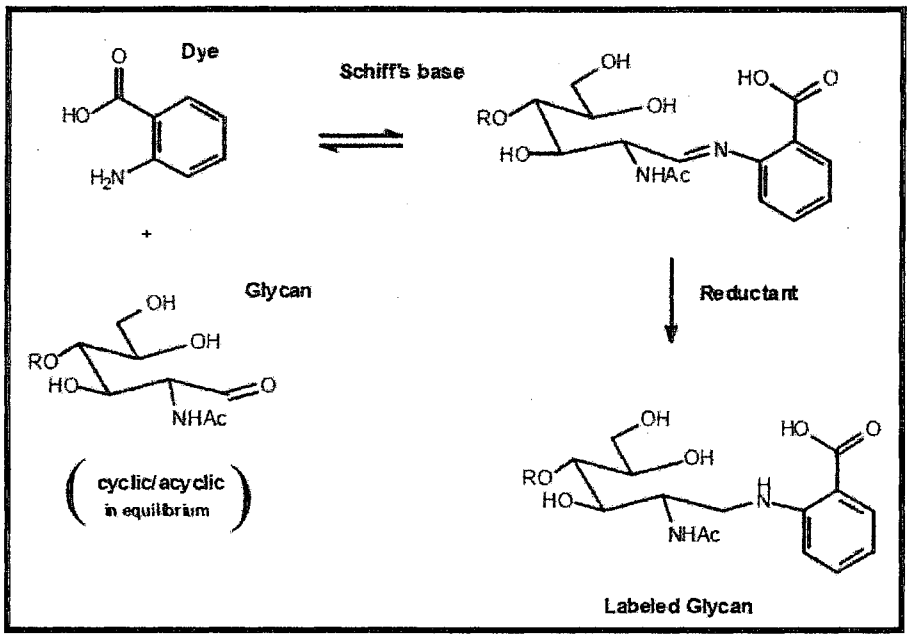
CHAPTER II

MICROWAVE-ASSISTED NON-REDUCTIVE RELEASE FOR O-GLYCAN ANALYSIS

Introduction

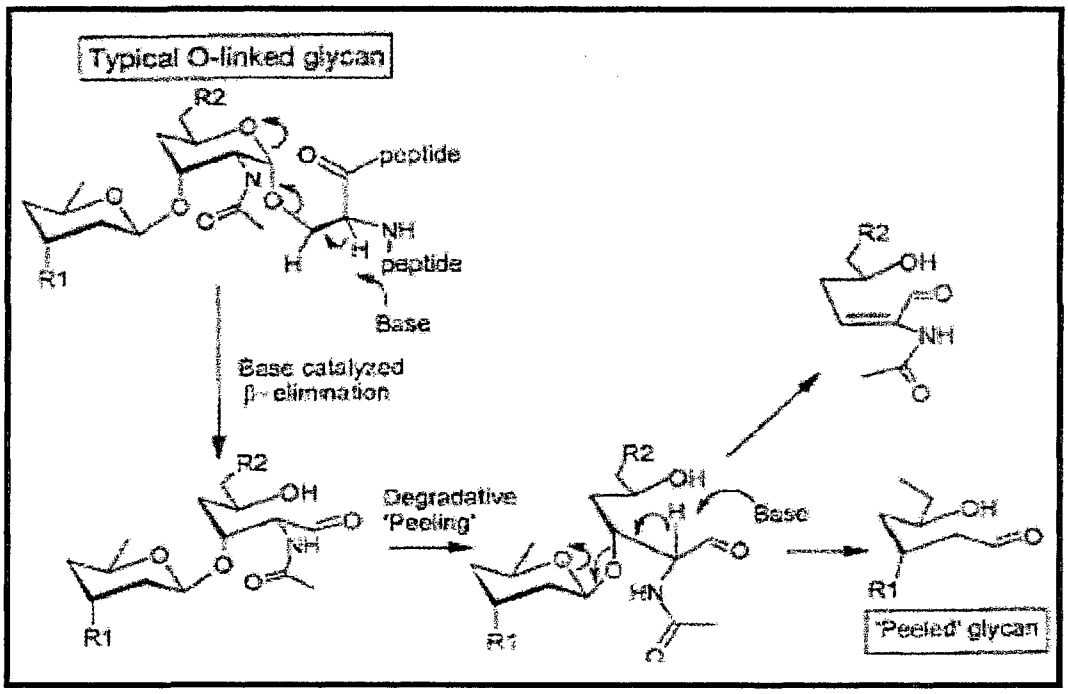
Analysis of free, unreduced, carbohydrates is difficult due to both the absence of a natural chromophore, and the presence of side reactions such as glycan degradation. Often the separation and analysis of carbohydrates is performed by high performance liquid chromatography (HPLC) or capillary electrophoresis(CE).[28] These methods are useful for separation and relative quantification and qualification, however for accurate compositional analysis, investigations of new or complex material, and determination of detailed oligosaccharide structure, mass spectrometric (MS) methods need to be used. Since carbohydrates do not contain natural chromophores sensitive to optical detection, and other detection methods such as those based on refractive index are not useful for complex or small sample sizes as they are not sufficiently sensitive[29], a label must be incorporated into the glycan to facilitate detection. Carbohydrate labeling is usually performed via reductive amination using a fluorescent tag such as 2-aminobenzamide (2-AB), 2-aminobenzoic acid (2-AA),

or 2-aminopyridine (2-AP). The reductive amination reaction scheme is shown in Scheme 2.1.[30]



Scheme 2.1: Labeling of unreduced glycans with 2-AA (Anthranilic acid). The glycan must have a free reducing end for the reaction to take place

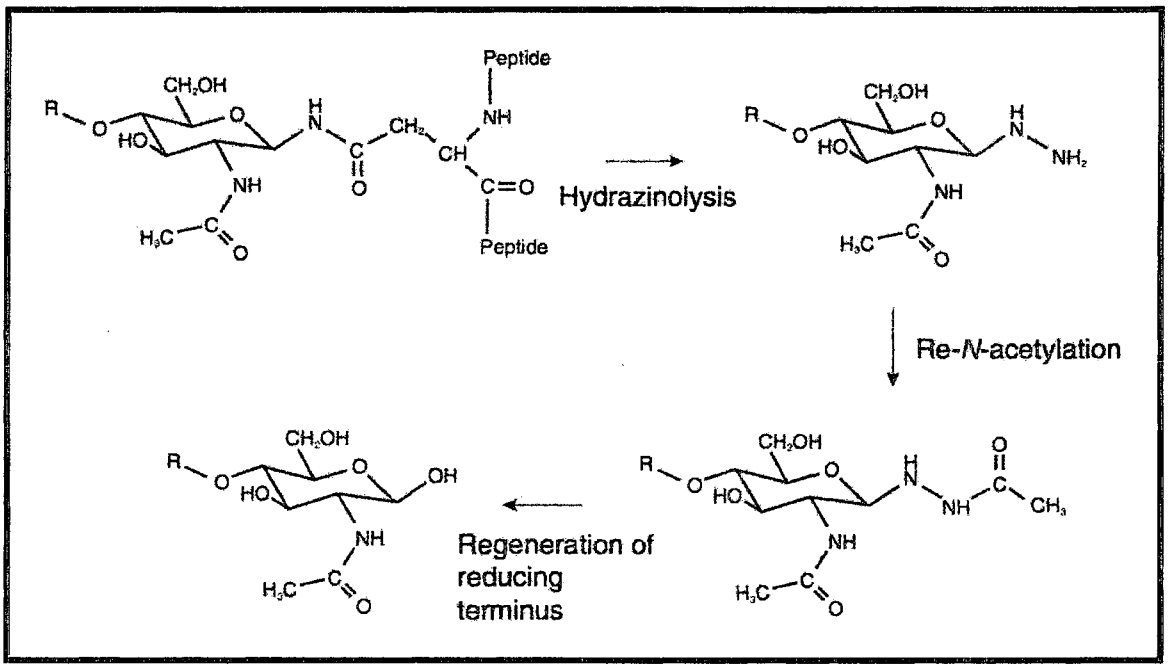
Reductive amination requires the glycan to be in its non-reduced form, therefore for an effective analysis by HPLC or CE the O-glycans must be released from the protein in a non-selective manner and remain in an unmodified and non-degraded form.[31, 32] Released carbohydrates in their non-reduced form exist as aldehydes (Figure 1.2). Further β-elimination, initiated at the reducing terminus, occurs due to the basic solution used for the release from the protein. This common degradation is known as peeling and is shown in Scheme 2.2. It is because of this reaction that the O-glycans are typically reduced *in situ* with β-elimination release and analyzed by MS.



Scheme 2.2: Base catalyzed β-elimination of O-linked glycans and subsequent degradative peeling of the non-reduced glycan

The most widely used non-reductive β-elimination O-glycan release strategy is hydrazinolysis. This method can release both N- and O-glycans at different temperatures for selective release of O-glycans at 60°Cfor 6 hrs and N-glycans at 100°Cfor a 4 hr reaction time.[33] The O-glycan release reaction occurs via a β-elimination due to the basic hydrazine (H_2N_2). Following release, the glycan may react with excess hydrazine to form a hydrazone derivative which must be re-acetylated, finally the reducing end is regenerated to obtain the O-glycan in its original form,[29] the hydrazinolysis reaction is outlined in Scheme 2.3. This method involves a tedious procedure and significant peeling by-products have been observed.[34] The release efficiency has never been carefully measured because the parent proteins are completely lost under these harsh conditions,

and the only effective way to be certain that all O-glycans are removed is to analyze the completely de-O-glycosylated peptide.



Scheme 2.3: Hydrazinolysis based non-reductive O-glycan release reaction scheme

Although anhydrous hydrazine is the most common non-reductive strategy for O-glycan release, other non-reductive methods have also been developed. Sodium hydroxide (NaOH) is effective in releasing O-glycans, however, rapid base hydrolysis of the peptide due to long reaction times, peeling of the glycans, as well as rigorous sample clean up lead to significant sample loss.[35]

Ammonium hydroxide (NH_4OH) as the releasing agent has been utilized to remove the glycan for O-glycosylation site studies with a 15 hr reaction time at 45°C.[36] As an extension of this method, saturated ammonium carbonate was added into the solution to protect the O-glycan from peeling.[37] Although this

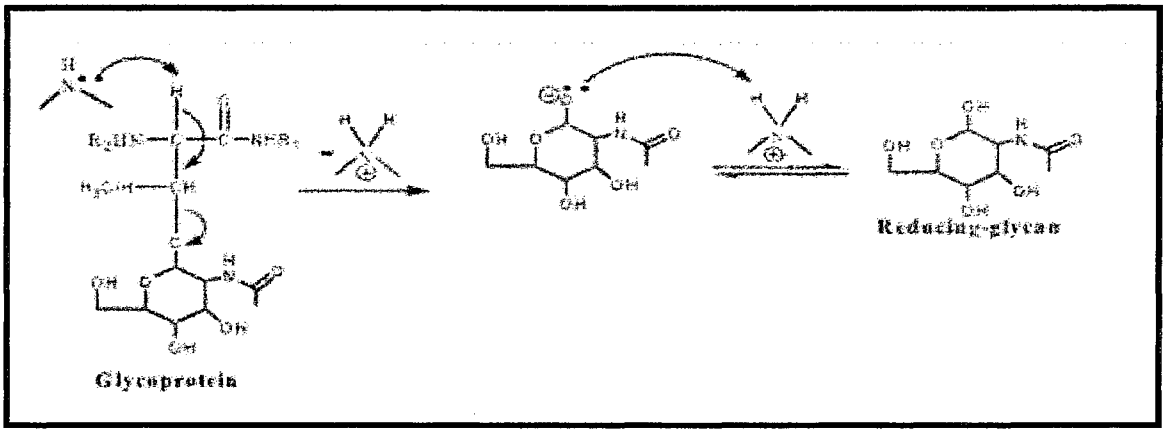
method is successful in releasing O-glycans for derivatization and electrophoretic separation,[38] no quantitative analysis was carried out and the release efficiency is questionable as the effect of the addition of ammonium carbonate has never been evaluated. Furthermore, significant peeling products have been detected possibly because ammonia is not efficient at protecting free glycans for these long reaction times.[39]

Primary organic amines such as methylamine, hydroxylamine, and ethylamine have been utilized as the base for O-glycan release at 45-50°C for 6-18 hrs. Peptide degradation occurred in all cases to varying degrees based upon reaction time and amine composition. Optimal reaction conditions of 70% ethylamine at 50°C for 18 hrs provided 70% release of non-reduced O-glycans with weak peptide degradation, however the extent of peeling was not evaluated.[40]

Ionic strong bases have also been employed to obtain non-reduced glycans using in-line flow systems to prevent side reactions, such as a polyvinyl difluoride membranes[41] and Poros R2 beads[42] immediately followed by neutralization with an acidic solution. A specific instrument, called the AutoGlycoCutter, has been developed to release O-glycans in as few as 3 min.[43] Although these methods have very short reaction times, they require special equipment, and the release efficiency has not been evaluated, but is predicted to be low.

Goals of the method

The goals of this method were to develop a new microwave-based non-reductive O-glycan release method, utilizing the short reaction time benefit of microwave radiation to obtain O-glycans in a non-reduced form with minimal peeling. An aqueous solution of dimethylamine (DMA) (40%, 8M) was chosen as the base for the release because DMA has the capability of both releasing the O-glycan, as shown in Scheme 2.4, and adding to the former O-glycosylation site (serine or threonine residue) for peptide sequencing and glycosylation site determination. The glycosylation site determination portion of this method will be discussed in Chapter III. It was previously shown that ammonium carbonate may protect the glycan from peeling, by forming a glycosylamine,[37] and therefore was used in this method. The use of aqueous DMA and ammonium carbonate provided for simple post-reaction sample clean up due to the volatile reagents, which is especially useful when dealing with limited quantities of biological samples.



Scheme 2.4: Proposed reaction mechanism for microwave-assisted non-reductive O-glycan release.

Experimental Methods

All microwave reactions were run in a CEM (Matthews, NC) Discover LabMate microwave equipped with an external infrared sensor for temperature feedback in closed vessel mode.

Materials

All solvents (HPLC grade) were purchased from Fisher (Fair Lawn, NJ), (all solutions used are reported as percents by volume unless otherwise noted). β-cyclodextrin, fetuin from fetal calf serum, ribonuclease A, trifluoroacetic acid (TFA), sodium hydroxide (NaOH), methyl iodide, dimethyl sulfoxide (DMSO), dithiothreitol (DTT), iodoacetamide (IAA), trypsin (proteomics grade), sodium borohydride, sodium cyanoborohydride, 2-aminobenzoic acid (2-AA), 9:1 2,5-dihydroxybenzoic acid: 2 -hydroxy-5-methoxybenzoic acid (super DHB), and 40% aqueous DMA were purchased from Sigma (Saint Louis, MO). Porous graphitic carbon (Carbograph 120/400) (PGC) and C18 solid phase extraction (SPE) columns were obtained from Alltech associates (Deerfield, IL).

Microwave-Assisted O-glycan Release

An aqueous solution of 50 mg bovine fetuin and 50 µg β-cyclodextrin was prepared, and portions of this mixture were used for all analyses. For each trial, an aliquot containing 1 µg of β-cyclodextrin and 1 mg fetuin was added to a 10 mL microwave reaction vessel with 500 µL 40% aqueous DMA saturated with ammonium carbonate (~250-300 mg). A small stir bar was added to equalize heating through the reaction. Five replicate reactions were run at 70°C for time

points ranging from 0 to 180 min in the microwave reactor. Once the reaction was complete, solvents were removed by repeat evaporations with water under a stream of nitrogen gas until no visible trace of ammonium carbonate remained. Further purification was performed on a PGC cartridge as described below.

Traditional Carlson Release

An aliquot of the sample mixture containing 1 mg fetuin and 1 µg cyclodextrin was dissolved in 1 mL of an aqueous solution of 50 mM NaOH/ 1M $NaBH_4$ in a glass tube. The reaction mixture was incubated at 50°C for 16 hrs with occasional venting of hydrogen gas. The reaction was terminated by addition of 1.5 mL acetic acid in an ice bath. Solvents were removed by evaporation in a SpeedVac. Borate esters were removed by repeat evaporations with 1% acetic acid in methanol. Further purification was performed on a PGC cartridge as described below.

Sample Clean Up

A PGC SPE column was used to purify and desalt the O-glycan samples. Dry PGC was washed with three sequential repetitions of 4 mL of each of the following solutions: (1) 1M NaOH; (2) H_2O; (3) 80% aqueous acetonitrile (ACN)/ 0.1% TFA; (4) 25% aqueous ACN/ 0.1% TFA; (5) 25% aqueous ACN; and (6) H_2O. The prepared PGC was loaded into a 1.5 mL reservoir column and equilibrated with 3 mL water. Dry samples were dissolved in water and applied to the column. The columns were each washed with 6 mL water and the O-glycans were eluted with 6 mL of 25% aqueous ACN/ 0.1% TFA then dried in a SpeedVac.

Permethylation

Since glycans containing sialic acid are difficult to quantify in positive ion mode MS, all released glycans were permethylated to obtain optimal mass spectrometry signals. Permehtylation was performed by the method developed by Ciucanu and Kerek.[44] Dry O-glycans were dissolved in 400 µL DMSO saturated with NaOH and vortexed for 15 min. To the reaction mixture, 100 µL methyl iodide was added and the mixture was vortexed for 75 min. The mixture was cooled in ice before the reaction was terminated by addition of 3 mL cold water. Glycans were recovered by repeat liquid-liquid extractions with dichloromethane and water. The dichloromethane layers were combined and the solvent was removed under a stream of nitrogen gas. The permethylated O-glycans were stored at -20°C until MS analysis.

Peptide Digestion

Ribonuclease A (1 mg) was dissolved in 200 µL 8 M urea/ 0.4 M ammonium bicarbonate and reduced with 20 µL of a freshly prepared 45 mM DTT solution at 50°C for 45 min. The solution was cooled to room temperature before alkylation by addition of 10 µL of a freshly prepared 100 mM IAA solution, which was incubated at room temperature, in the dark, for 15 min. The reduced and alkylated protein sample was diluted with 690 µL water then digested with trypsin 1/20 (wt/wt) overnight at 37°C. The reaction was terminated by addition of several drops of TFA, then the samples were dried in a SpeedVac. Dry peptides were then dissolved in 1 mL of 0.1% aqueous TFA and applied to a C18 SPE

column which was conditioned with ACN and equilibrated with 0.1% aqueous TFA. The column was washed with 6 mL 0.1% aqueous TFA, and the peptides were eluted with 6 mL 60% aqueous ACN/ 0.1% TFA and dried in a speed vac. The peptides were dissolved in 0.1% aqueous TFA prior to MS analysis.

Reductive Amination

A 500 µg sample of maltoheptaose was subject to one hour 70°C in microwave reactor with 500 µl 40% aqueous DMA saturated with ammonium carbonate. After clean up by a PGC column as described previously, the sample was dried completely. Labeling with 2-aminobenzoic acid (2-AA) was achieved by the addition of 5 µL of a 0.44 M 2-AA solution in 30% acetic acid in DMSO and 5 µL 0.95 M sodium cyanoborohydride in 30% acetic acid in DMSO. The mixture was incubated at 60°C for three hrs. The reaction was terminated by evaporation of reagents under nitrogen gas. The sample was dissolved in 1 mL water and applied to a second PGC column, dried, then stored at -20°C prior to MS analysis.

MS Methods

Electrospray ionization mass spectrometry (ESI MS) was performed on a linear ion trap mass spectrometer (LTQ, ThermoFinnigan, San Jose, CA) equipped with a nanoelectrospray source, 0.30 µL/min flow rate at 200°C capillary temperature in positive ion mode with a spray voltage of 1.4kV.

Matrix-assisted laser desorption ionization time of flight mass spectra (MALDI-TOF MS) were recorded using a Kratos-Axima Curved Field Reflectron mass spectrometer (Manchester, UK) The instrument was calibrated externally

with peptide and carbohydrate standards. Positive ion mass spectra were acquired in reflectron mode using super DHB (10 mg/mL in 50% aqueous ACN) as the matrix. 1.0 µL sample and 1.0 µL matrix were co-crystalized onto a stainless steel target plate. Post extraction was set to optimize for ions m/z 1500 with a laser power of 135. 500 laser shots were fired in 10 shot units over the target surface.

Results and Discussion

Determination of Ideal Microwave Reaction Time

Bovine fetuin is a well-characterized glycoprotein containing acidic (sialic acid containing) N- and O-glycans.[45] This glycoprotein standard was utilized to determine ideal microwave conditions for non-reductive O-release. β-cyclodextrin was included in the reaction mixture as the internal standard to measure the relative release efficiency. β-cyclodextrin does not contain a free reducing end and therefore cannot participate in degradative peeling reactions, however it contains all other structural features of common oligosaccharides. O-glycans were released from fetuin under microwave heating utilizing aqueous DMA saturated with ammonium carbonate at 70°C for reaction times from 0-180 min.

A representative ESI mass spectrum after a 60 min reaction, is shown in Figure 2.1. Two unreduced O-glycans were observed; H1N1A1 (called O-1), m/z 879.3 $[M+Na]^+$ and 857.2 $[M+H]^+$, and H1N1A2 (called O-2) m/z 1240.2 $[M+Na]^+$, The internal standard, β-cyclodextrin, was observed at m/z 1451.2 $[M+Na]^+$, and

the major peeling product, H1A1 (called P-1), was observed at m/z 634.2 $[M+Na]^+$(H=Hexose, N=HexNAc, A=Sialic acid).

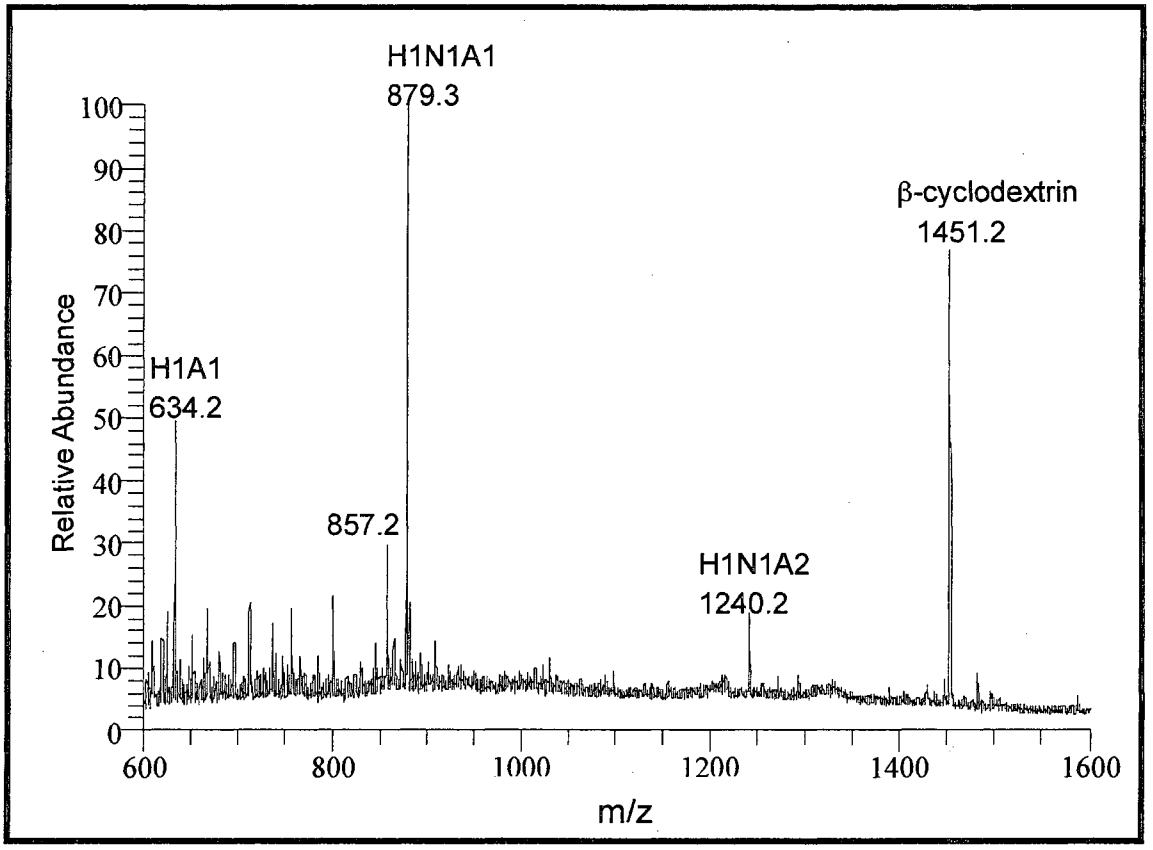


Figure 2.1: Representative spectrum: ESI MS of the released O-glycans from bovine fetuin after a 60 min microwave reaction at 70°C with aqueous DMA saturated with ammonium carbonate (H=Hexose, N=HexNAc, A=Sialic acid)

Time-release data, over a 5 replicate average, of the ESI MS relative abundances of unreduced fetuin glycan O-1 and its major peeling product P-1 relative to β-cyclodextrin are plotted in Figure 2.2. Longer microwave reaction times lead to more O-glycan released, however, they also lead to increased peeling. Poor reproducibility, represented by standard deviations, in these results was attributed to the saturation of the DMA solution with ammonium carbonate.

At the time of these experiments, it was believed that ammonium carbonate only functioned to protect the glycan and therefore the exact amount was not reproduced from trial to trial, so long as the solution was saturated. However, as explained in Chapter III, it was later determined that ammonium carbonate significantly affects the release and hence variability in trials may occur. Since peeling increased with time, a trade-off exists between optimal release and minimal peeling when choosing the ideal reaction conditions.

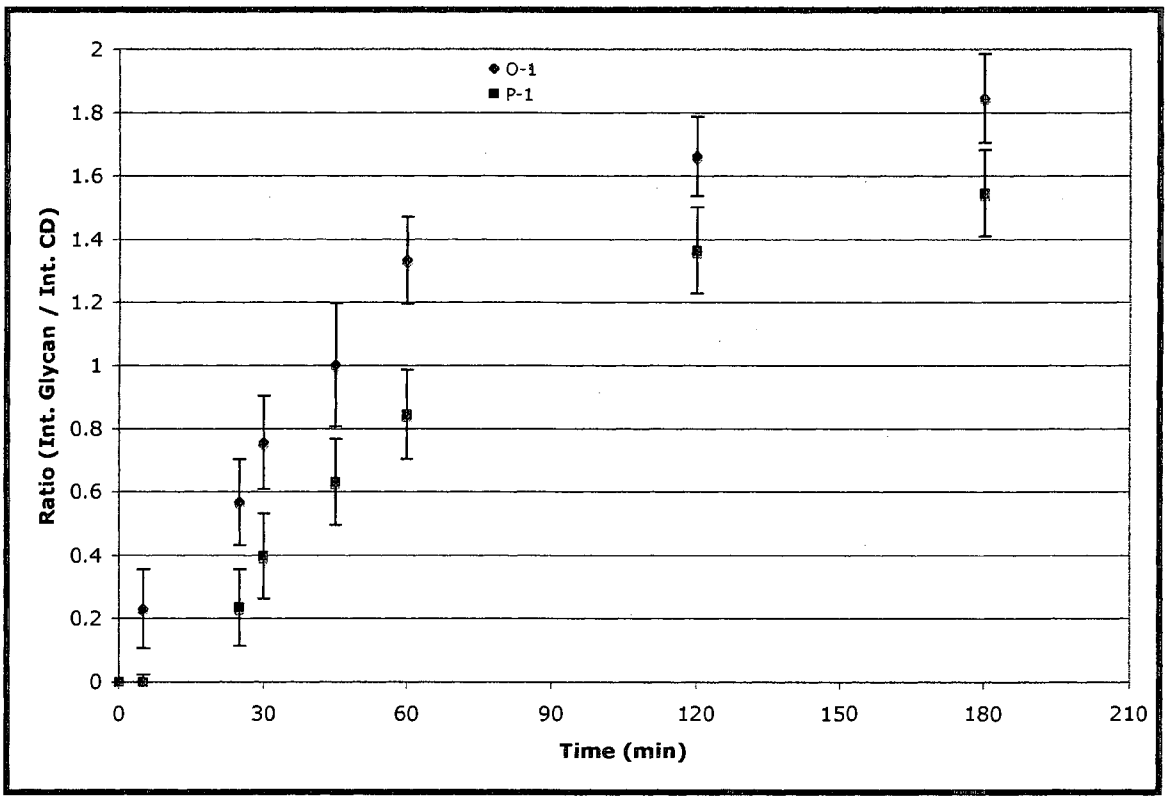


Figure 2.2: Time study of DMA based non-reductive microwave-assisted O-glycan release on bovine fetuin at 70°C. Average ESI MS peak intensity of non-reduced permethylated O-1 (H1N1A1) glycan and P-1 (H1A1) major peeling product relative to β-cyclodextrin internal standard is plotted. Error bars represent standard deviations. Greater than 2 hrs would be necessary for complete release.

After prolonged heating in the microwave, O-glycans still remained on the peptide. Such long reaction times begin to diminish the benefit of microwave radiation to this method, so it was determined that a one hour maximum reaction time would be utilized. Further optimization studies on this reaction will be described in Chapter III.

Figure 2.3 is a comparison of the results of one hour microwave-assisted non-reductive release to a 16 hr classical heating block NaOH/ $NaBH_4$ release (this classical reductive release method, discussed in Chapter IV, is currently accepted as the most efficient O-glycan release method). The glycans observed in both spectra were the same, in Figure 2.3B, the glycans were unreduced and in Figure 2.3A they were reduced, hence the 16 Da mass difference after permethylation. The difference between the two spectra is the relative abundance of the internal standard peak (m/z 1451.5). In the classical method it is significantly smaller than in the microwave method, suggesting that more O-glycans were released by the classical method. It should also be noted that peeling products (m/z 650/634) were obtained from the classical release method as well as the new microwave based method, however in lower yield. Even in the best available method, peeling is unavoidable.

Efficiency calculations were performed by finding the ratio of peak intensity of O-1 glycan to β-cyclodextrin, where a higher ratio indicated more O-glycan was released. Yields from the microwave-assisted method were less than 10%

that of the classical method. However, non-reduced glycans were able to be obtained.

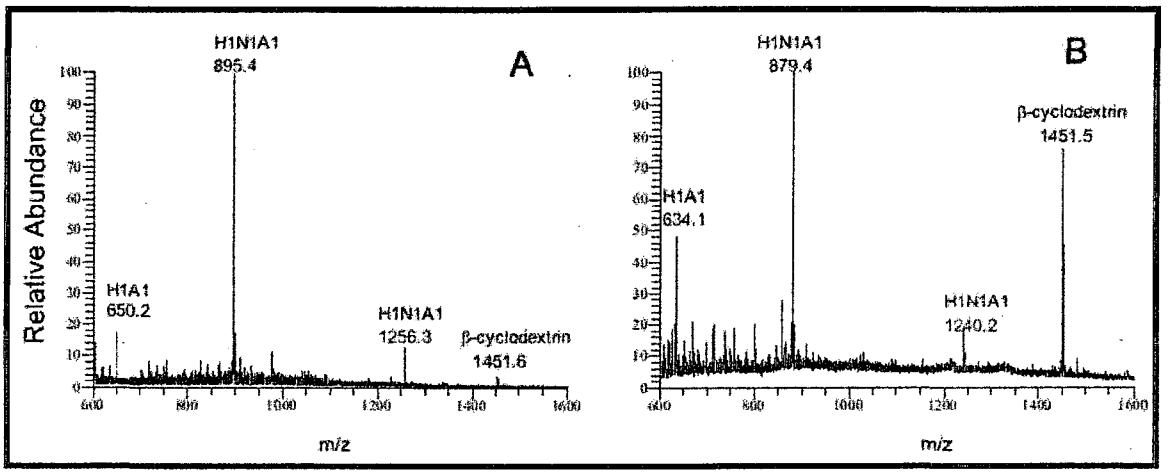


Figure 2.3. Comparison of O-glycan release efficiency of the two methods on bovine fetuin relative to β-cyclodextrin internal standard. (A) classical heating block NaOH/NaBH4 16 hr 50℃ (B) Microwave DMA/ ammonium carbonate 1 hr 70℃. (H=hexose, N=HexNAc, A=Sialic acid)

Effect of the Method on Peptides

Ribonuclease A is a non-glycosylated protein with tryptic peptides shown in Table 2.1. The tryptic peptide sequence listed was obtained from the UniProtKB/Swiss-Prot database (entry name: RNAS1_BOVIN). A MALDI mass spectrum of a sample of ribonuclease A after reduction with DTT, alkylation with IAA, and digestion with trypsin is shown in Figure 2.4A. The five high mass peptides were observed, corresponding to 72% sequence coverage; peptides less than 700 Da were not clearly detected due to matrix and other low mass interferents. This sample was then exposed to one hour 70℃ microwave irradiation with aqueous DMA saturated with ammonium carbonate. Comparisons

between the sample exposed to the microwave conditions and an unreacted sample, shown in Figure 2.4B and Table 2.2, demonstrated that the one hour microwave heating method had no adverse effect on the peptide backbone as all the same tryptic peptides were detected after the microwave reaction.

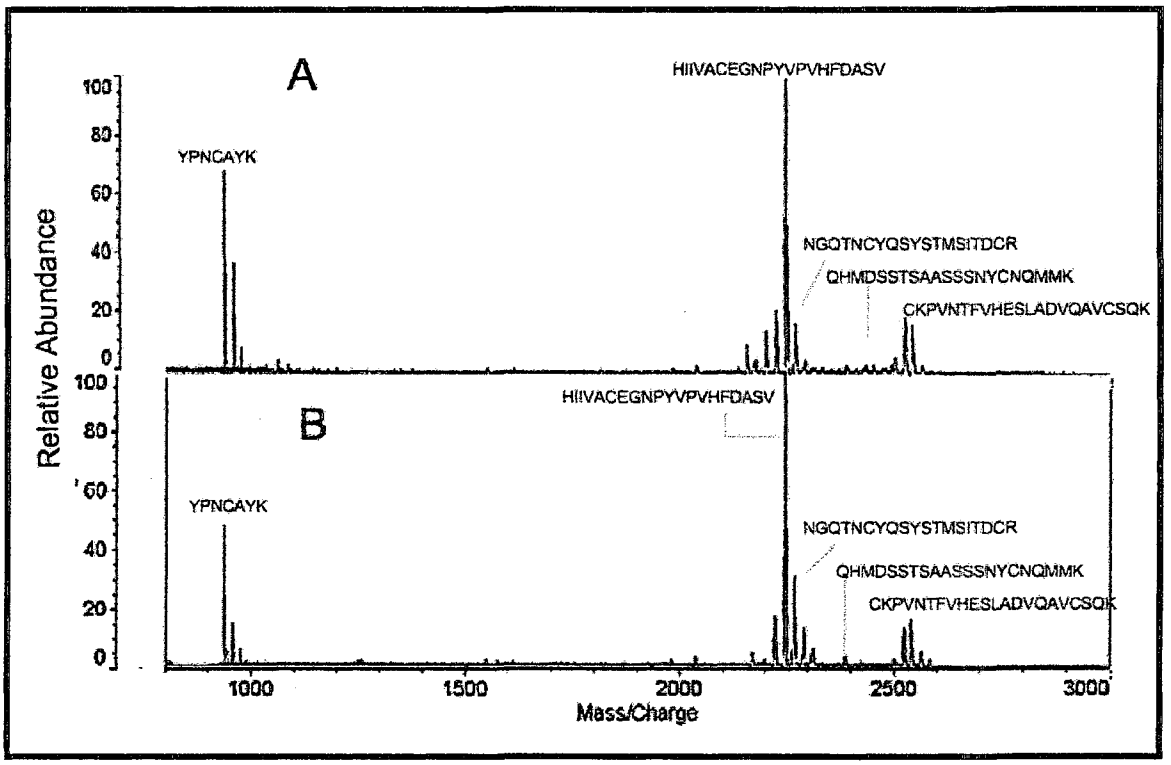


Figure 2.4: MALDI MS of the tryptic peptides of ribonuclease A (A) Without microwave heating and (B) After one hour microwave heating in DMA saturated with ammonium carbonate at 70°C.

Table 2.1: Ribonuclease A tryptic peptides before and after one hour microwave radiation at 70°C in an aqueous DMA/ ammonium carbonate mixture. No adverse effects on the peptides were seen after the reaction.(X=peptide was observed in the spectrum)

Tryptic peptides	$[M+Na]^+$	Tryptic peptides before reaction	Tryptic peptides after reaction
CKPVNTFVHESLADVQAVCSQK	2539.22	X	X
QHMDSSTSAASSSNYCNQMMK	2386.92	X	X
NGQTNCYQSYSTMSITDCR	2377.89	X	X
HIIVACEGNPYVPVHFDASV	2246.08	X	X
YPNCAYK	937.40	X	X
TTQANK	684.34		
ETGSSK	630.28		
ETAAAK	612.31		
NVACK	613.32		
NLTK	497.28		
FER	473.22		
DR	312.13		
SR	284.14		
K	169.11		

Although a quantitative analysis was not done at the time, it does not appear that any peptide degradation or unfavorable side reactions occurred as no new peaks were seen in the spectra after the reaction. Therefore this non-reductive method was determined to have no adverse effect on the protein backbone and may be utilized for site determination or to remove glycans for further protein analysis.

Effect of the Method on the Reducing End of the Glycan

Preserving the reducing end of the O-glycan was another goal of this method. Results from bovine fetuin indicated that the reducing end was intact based on the mass difference between the released glycans from the two methods, as shown in Figure 2.3. To prove that the integrity of the reducing end was not compromised, a sample of maltoheptaose (Glu_7), an oligosaccharide with a free reducing end, was subject to the microwave-assisted release conditions and subsequently labeled by reductive amination with 2-AA. ESI MS results indicated, by mass shift, that the 2-AA label was incorporated on maltoheptaose after the microwave reaction. The m/z of unlabeled maltoheptaose is 1175 for the sodium adduct. Figure 2.5 shows the 2-AA labeled maltoheptaose in both its protonated and sodium adduct forms, m/z 1274.4 and 1296.3 respectively, indicating a mass shift corresponding to the 2-AA label. This successful labeling demonstrated that the method has no adverse effect on the free reducing end of the glycan.

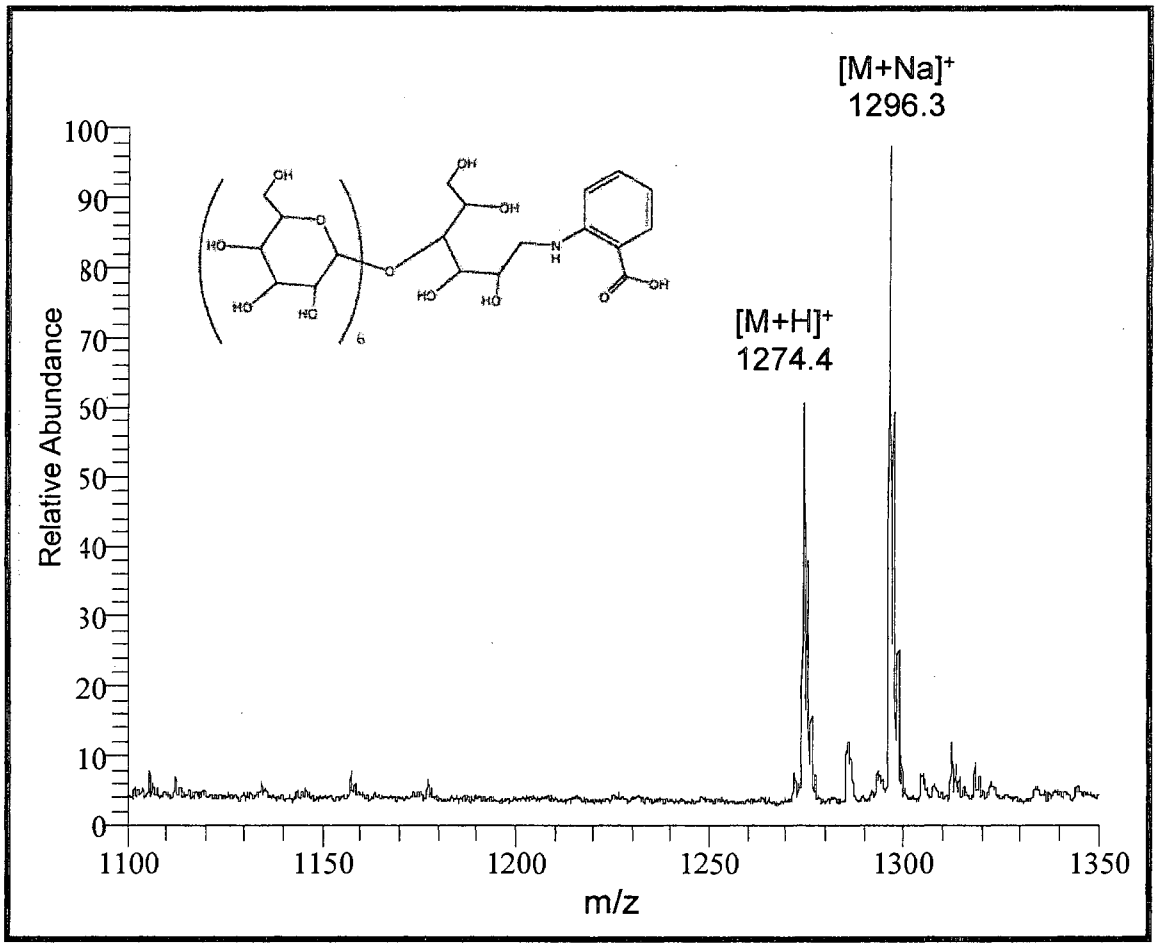


Figure 2.5: ESI MS of 2-AA labeled maltoheptaose following a one hour 70°C microwave incubation in DMA/ ammonium carbonate. The labeling reaction was successful indicating no adverse effect on the reducing end

Summary

A microwave-assisted non-reductive O-glycan release method was developed. The optimal reaction conditions were: 70°C in a microwave reactor for one hour with 40% aqueous DMA saturated with ammonium carbonate. Under these conditions, no damage to the peptide backbone or the glycan reducing end was observed. However, release efficiency compared to the

classical $NaOH/NaBH_4$ release with a heating block (Figure 2.4) was not as efficient. Lower yield could potentially be due to either increased peeling in basic solution (inefficient protection from ammonium carbonate) or simply inefficient release, or, most likely a combination of the two. Since the release was not as effective as expected, small glycopeptides were obtained and utilized to further understand and optimize the reaction, this method is described in Chapter III.

CHAPTER III

MICROWAVE-ASSISTED COMPLETE DE-O-GLYCOSYLATION AND APPLICATIONS TO GLYCOSYLATION SITE DETERMINATION

Introduction

When investigating O-glycans from unknown samples or when the goal is a quantitative comparison of different disease states, it is important to have a complete and efficient O-glycan release method. However, knowledge of the glycan structure is only a portion of overall glycomics; determination of the site of carbohydrate attachment on the protein backbone is required to thoroughly understand function. However, glycosylation site determination is hindered since detection of a glycopeptide in a complex peptide mixture is a challenging task.

When utilizing common ionization methods for MS such as MALDI or ESI, glycopeptides are difficult to detect due to the suppression of the ion signal by non-glycosylated peptides. The reason for this is that the non-polar side chains on amino acid residues produce better ion signals when compared to the polar nature of glycopeptides.[46] Furthermore, when glycopeptides are subject to collision-induced dissociation (CID) fragmentation, the carbohydrate

fragmentations are often the major ions in the MS^n spectra thus it is difficult to identify the glycosylation site.

Statistical methods have been developed to asses the probability that a certain peptide sequence contains an O-glycosylation site. Some trends for O-glycosylation sites have been observed, such as the surrounding amino acids are often serine, threonine, or proline rich domains, and this region has few charged residues, especially close to the glycosylation site. A predictive tool has been developed by the Center for Biological Sequence Analysis at the Technical University of Denmark for determining the chances of site occupancy, by observing the +/- 5 amino acid area around a potential glycosylation site: www.cbs.dtu.dk/services/NetOGlyc.[47] However, knowledge of the amino acid sequence of interest is required, and no structural information about the glycan can be obtained.

The development of chemical methods to aide in O-glycosylation site determination have shown that ammonium hydroxide as a base can release the O-glycan with subsequent addition of NH_3 across the double bond forming an ammonia-peptide adduct, which labeled the glycosylation site for further studies.[36] However, NH_3 adducts may be difficult to distinguish because the mass difference between ammonia and water (as in a native peptide) is only 1 Da. Additionally, the 40 hr reaction time associated with this method is impractical for high-throughput laboratories. Larger primary amines were introduced in an attempt to raise the mass difference of the adduct peptide, however low yields and/or moderate peptide degradation existed.[40]

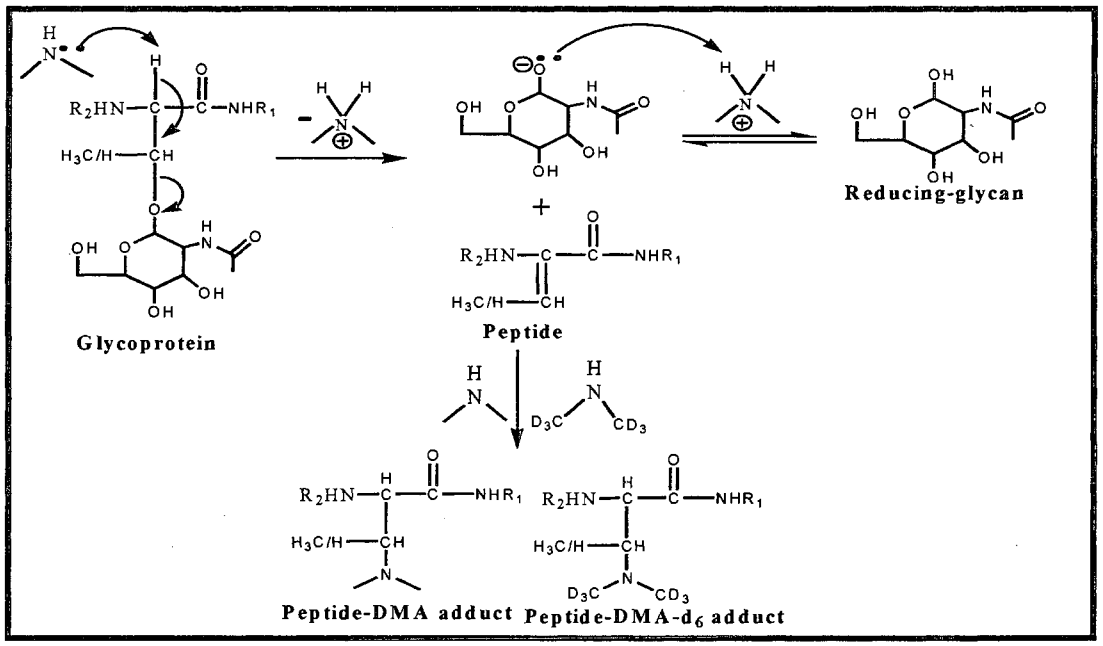
To circumvent the suppression effects in MS, isolation and enrichment of glycopeptides by affinity chromatographic methods has been employed with the use of lectins. Lectins are proteins which bind carbohydrates with high affinity and specificity, and are involved in many cell-cell interactions and signaling processes. For example, a well known lectin of the influenza virus, hemagglutinin, is essential for viral binding to host cells containing terminal sialic acids.[48]

Externally, the properties of lectins may be exploited for use as carbohydrate separators. A popular lectin for O-link glycan studies is jacalin, a lectin derived from the jackfruit. Jacalin has affinity for some common O-glycans such as the T-antigen (an O-glycan often associated with tumors)but does not have affinity for the sialyated derivative.[49] Many lectins exist with overlapping and specific binding requirements. When working with unknown and complex samples, it is often ineffective to utilize only one type of lectin due to the specific binding properties of the lectin and the unknown complex structure of the glycan.[50,51]

Another, potentially more universal, carbohydrate enrichment strategy is use of boronic acids. At high pH, boronic acids bind *cis*-diols, which may be eluted under acidic conditions.[52] Recently, boronic acids immobilized on magnetic beads as well as boronic acid functionalized mesoporous silica have shown promise as microscale glycoprotein separation devices.[53,54]

If the glycopeptide is successfully enriched, it is possible to obtain mass spectra before and after de-O-glycosylation and by mass shift, the composition of

the O-glycan may be determined. However, to confirm the specific site of glycosylation, MS^n methods need to be used. Investigations of glycoproteins by CID MS are hindered as the major fragmentations observed are losses of the sugar chain due to the lability of the peptide-glycan bond, and few peptide backbone cleavages are observed. Electron capture dissociation and electron transfer dissociation are able to generate glycosylated peptide fragment ions, however these instruments are not available in our laboratory at this time. The method developed in this chapter aimed to provide a label on the glycosylation site so it may be determined by CID MS. In addition to being the releasing agent, DMA adds to the peptide backbone, providing a stable label for glycosylation site determination, as shown in Scheme 3.1.



Scheme 3.1: Proposed reaction mechanism for microwave-assisted de-O-glycosylation and formation of DMA-peptide adducts

Goals of the method

The goal of this work was to develop a method for de-O-glycosylation which could be utilized after glycopeptide enrichment which quantitatively released O-glycans and labeled the peptide for glycosylation site determination. The releasing agent was aqueous DMA. Utilizing small standard glycopeptides, optimal time, temperature, and pH of the reaction solution were determined. Additionally, the use of $DMAd_6$ was investigated as well as the methods effects on various peptides. Since glycosylation site was of interest here, the O-glycan was not further investigated.

Experimental Methods

All microwave reactions were run on a CEM (Matthews, NC) Discover LabMate microwave equipped with an external infrared sensor for temperature feedback in closed vessel mode.

Materials

Two synthesized peptides were obtained from Dr. Hart (Johns Hopkins Medical School). The O-glycosylated and unglycosylated peptide sequences are shown in Fig 3.1. The unglycosylated peptide (further referred to as CTD peptide) served as an internal standard, while the O-glycosylated peptide (further referred to as OCTD peptide) was utilized for the release study. These peptides were chosen because their sequence resembles that of common O-glycopeptides, consisting of the amino acids serine, threonine, and proline.

Dimethyl-d6-amine hydrochloride 99%D was purchased from Sigma (Saint Louis, MO). C18 Zip-Tips were purchased from Millipore (Billerica, MA). All other materials utilized in this chapter were described in Chapter II: Experimental Methods.

A

Tyr-Ser-Pro-Thr-Ser-Pro-Ser-Lys

Tyr-Ser-Pro-Thr-Ser-Pro-Ser

O-GlcNAc

B

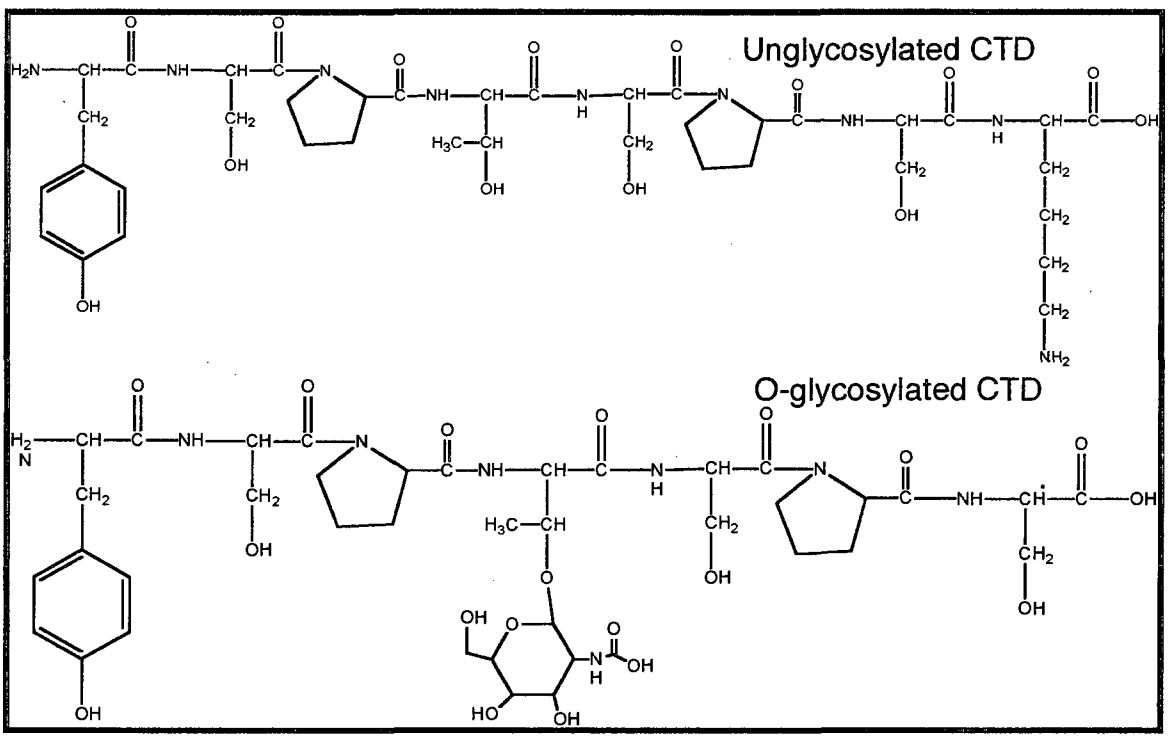


Figure 3.1: Peptides utilized for microwave optimization studies; (A) three letter code representation (B) Structural representation

Optimization of Microwave-Assisted De-O-glycosylation

A reaction mixture was prepared by combining equimolar amounts of unglycosylated (CTD) and O-glycosylated (OCTD) peptides in water, portions of this reaction mixture were used for each trial. An aliquot containing 50 µg each of

CTD and OCTD was added to 500 µL 40% aqueous DMA in a 10 mL microwave reaction tube and a small stir bar was added to equalize heating throughout the reaction. For pH optimization, the indicated amount of ammonium carbonate was added to the reaction mixture and the microwave was set at 70°C for one hour. Reaction temperature optimization was performed with a reaction time of one hour, and time optimization was performed with a reaction temperature of 70°C.

Once the reaction was complete, reagents were removed by repeat evaporations with water under a stream of nitrogen gas until no visible residue remained. Samples were dissolved in 50% aqueous methanol and ESI MS spectra were obtained as described.

Protein Digestion

Ribonuclease A protein digestion was accomplished as described in Chapter II: Experimental Methods.

Effect of DMA on the Peptide

After reduction, alkylation, and tryptic digestion, the peptides from ribonuclease A were subject to the indicated conditions in the microwave. After the reaction, samples were passed through a C18 Zip-Tip equilibrated with 0.1% aqueous TFA, the peptides were eluted with 60% aqueous ACN/ 0.1% TFA

Heating Block Comparison

An aliquot of the reaction mixture containing 50 µg each of CTD and OCTD was mixed in a glass tube with 500 µL 40% aqueous DMA solution. The tube was capped and placed into a heating block set at 70°C for a reaction time

of one hour. Following the reaction, sample clean up was the same as described for the microwave samples.

DMAd$_6$ Labeling

Neutralization was accomplished by dissolving 0.008 mol Dimethyl-d$_6$-amine hydrochloride in 1 mL 0.008 M NaOH. A 50 mol% DMA /DMAd$_6$ solution was prepared by combining 1 mL of 40% aqueous DMA solution with the neutralized DMAd$_6$ solution. 500 µL of the 50% DMA/ DMAd$_6$ solution was utilized for the labeling experiments.

MS Methods

Electrospray ionization mass spectrometry (ESI MS) and Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) were performed as described in Chapter II: Experimental Methods.

Results and Discussion

Optimization of Microwave Conditions

To establish zero percent release conditions, an unreacted mixture of CTD and OCTD peptides was analyzed by ESI MS, a representative spectrum is shown in Fig 3.2. There were two ions which corresponded to OGlcNAc CTD: m/z 963.3 $[M+Na]^+$ and 985.2 $[M-H+2Na]^+$ and three ions which corresponded to CTD: m/z 866.3 $[M+H]^+$, 888.3 $[M+Na]^+$, and m/z 910.2 $[M-H+2Na]^+$. Importantly, no ions corresponding to the loss of GlcNac from the glycopeptide were present in the zero minute standard, so it can be assumed in

all further spectra that the ions observed correspond to chemical reaction products only.

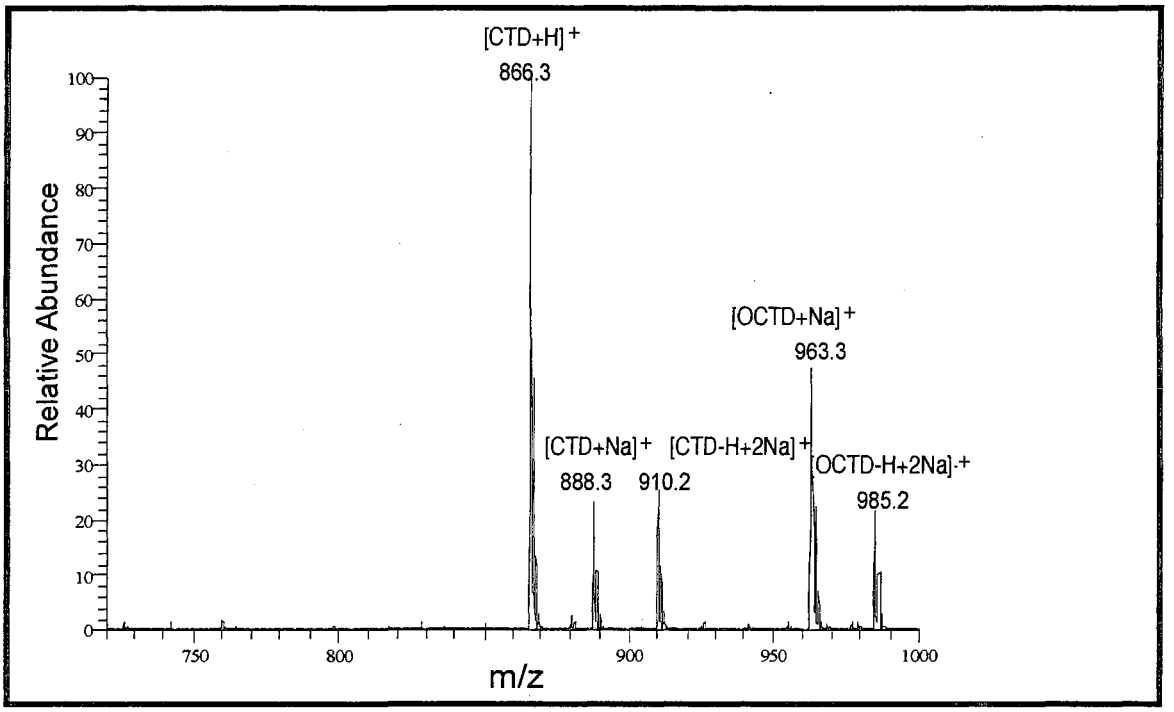


Figure 3.2: ESI MS of CTD and O-GlcNAc CTD (OCTD) peptides, zero min reaction time. No ions corresponding to de-O-glycosylated peptide were present.

To show that the unglycosylated CTD peptide was an effective internal standard, it was subject to one hour heating at 70°C in the microwave with 40% aqueous DMA. In Figure 3.3, three ions were observed that corresponded to the CTD peptide. No evident degradation of the peptide or other side products were present when compared to an unreacted standard.

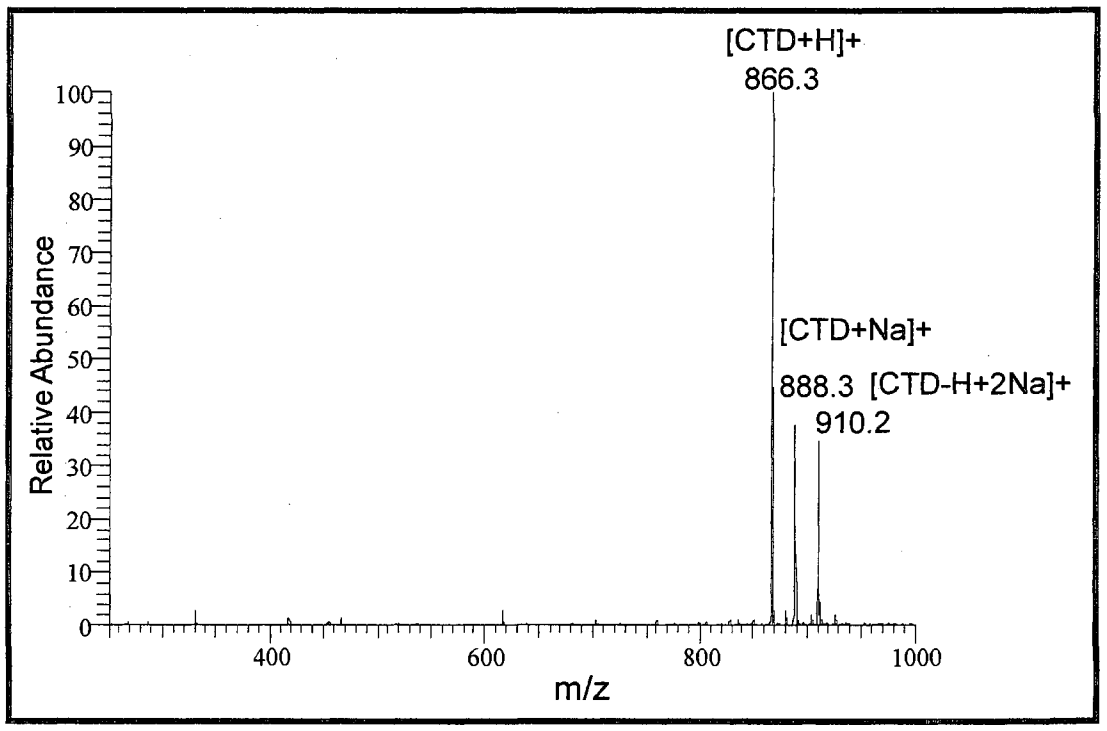


Figure 3.3: ESI MS of unglycosylated CTD peptide after one hour in the microwave at 70°C with aqueous DMA. No ions corresponding to peptide degradation were detected.

The two peptides were reacted in the microwave reactor at 70°C with aqueous DMA saturated with ammonium carbonate (the conditions as described in Chapter II) for various time points. Figure 3.4 outlines the release dependency on reaction time in the microwave. Product ions indicating the loss of GlcNAc from the OCTD peptide and formation of a double bond, and adduction of either ammonia or DMA were observed. The structures of these ions are described in Table 3.1.

As expected based on the non-reductive bovine fetuin O-glycan release data in Chapter II, the reaction was not efficient in releasing O-glycans. This

result was confirmed in the mass spectra of the reaction products, a high abundance of starting material OCTD and relatively low abundance of the de-O-glycosylated peptide was apparent after the one hour 70°C microwave reaction. From these data it appeared that reaction times greater than three hrs would be necessary to efficiently release all O-glycan from the peptide. However, it is known that long reaction times lead to hydrolysis of both the glycan and the peptide.

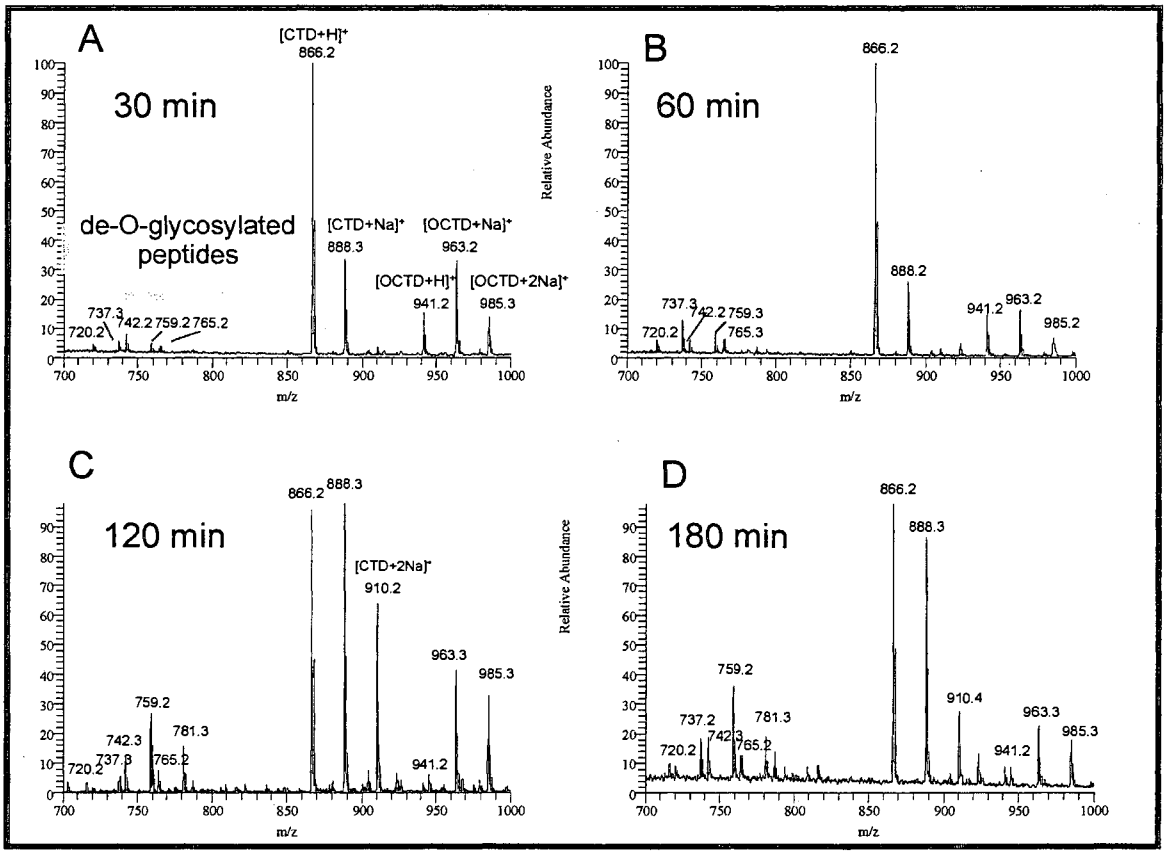


Figure 3.4: Effect of microwave reaction time on de-O-glycosylation of the OCTD peptide, ESI MS of CTD and OCTD (A) 30 min; (B) 60 min; (C) 120 min; (D) 180 min at 70°C with 40% aqueous DMA saturated with ammonium carbonate.

Table 3.1: Structures of the de-O-glycosylated reaction product ions observed in the ESI mass spectra after microwave-assisted DMA/ ammonium carbonate release

m/z	Structure
720.2	[OCTD-GlcNAc+H]$^+$
737.3	[OCTD-GlcNAc+NH_3+H]$^+$
742.3	[OCTD-GlcNAc+Na]$^+$
759.2	[OCTD-GlcNAc+NH_3+Na]$^+$
765.2	[OCTD-GlcNAc+DMA+H]$^+$
781.3	[OCTD-GlcNAc+NH_3-H+2Na]$^+$

The explanation for the ineffective release is the addition of ammonium carbonate to the reaction mixture. Although ammonium carbonate may prevent peeling of the glycan, as we and others have noted, it will also hinder the release by lowering the pH of the reaction solution. β-elimination of the O-glycan relies on strongly basic conditions, the pH of 40% aqueous DMA was ~12.5, however once saturated with ammonium carbonate the pH decreased to ~11.0. This decrease in pH upon saturation may explain the ineffective release.

Figure 3.5 confirms the role of ammonium carbonate (pH) in the release reaction. It is clear that with increasing concentrations of ammonium carbonate, the release reaction was hindered, also there was more chemical noise in the spectra. There was a relatively high abundance of de-O-glycosylated peptides in the sample that was reacted with 50 mg ammonium carbonate, Figure 3.5A, compared to the relatively low abundance in the sample with a large amount, Figure 3.5D. Figure 3.5A shows that with the addition of only 50 mg ammonium

carbonate to the reaction mixture a significant amount of O-glycan release occurred, only two ions (m/z 945 and 985) which correspond to intact OCTD starting material remained and at small ion abundances. However, formation of a series of ammonia and DMA peptide adducts (Figure 3.5, Table 3.1) is unfavorable especially in a complex or unknown mixture. Ammonia adducts are unfavorable for unknown peptides because the difference between an ammonia adduct and an unglycosylated peptide, which would simply have H_2O across the bond, have a mass difference of 1 Da. Such mass differences are difficult to detect on mass spectrometers with low resolution and mass accuracy.

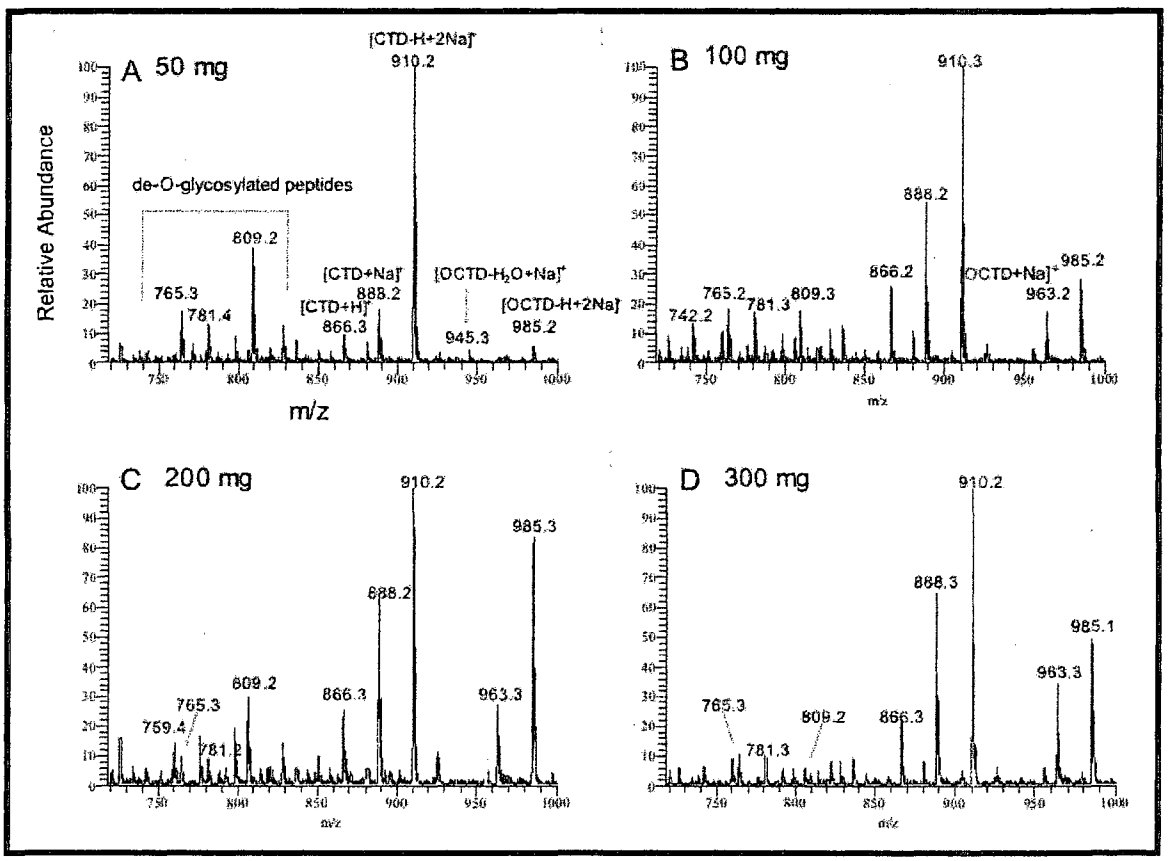


Figure 3.5: Effect of ammonium carbonate concentration on aqueous DMA microwave-assisted de-O-glycosylation reaction. ESI MS of CTD and OCTD peptide reaction products after addition of (A) 50 mg; (B) 100 mg; (C) 200 mg; (D) 300 mg ammonium carbonate, 1hr reaction at 70°C

The addition of 100 mg or more of ammonium carbonate to the reaction mixture resulted in significant hindering of the O-release. It can be speculated that amounts less than 100 mg (not saturated) will be inefficient at protecting the glycan from peeling, therefore it was determined that ammonium carbonate has no benefits to the reaction. Additionally, since glycosylation site determination was of primary interest in this method, removal of ammonium carbonate simplified the product field.

Figures 3.6 and 3.7 show the release of GlcNAc from the OCTD peptide depending on temperature with aqueous DMA alone. For these trials, a reaction time of one hour was chosen as this was previously determined as the maximum reasonable time to utilize the microwave advantage.

Quantification was performed in a similar manner to that reported by Rebecchi and coworkers.[55] First the relative ion abundances of the first three isotopic peaks of each composition were summed. Next, all the abundancies which corresponded to the same peptide but contained different charge carriers or adducts were summed to give the intensity. A total of three intensities were obtained for each spectrum which corresponded to; CTD peptide, OCTD peptide, and O-glycosylated product. For each trial, the ratio between the intensities of the intact OCTD starting material and the CTD internal standard, and the ratio between the intensities of the de-O-glycosylated peptide and the CTD internal standard was calculated, averaged, and plotted in Figure 3.6.

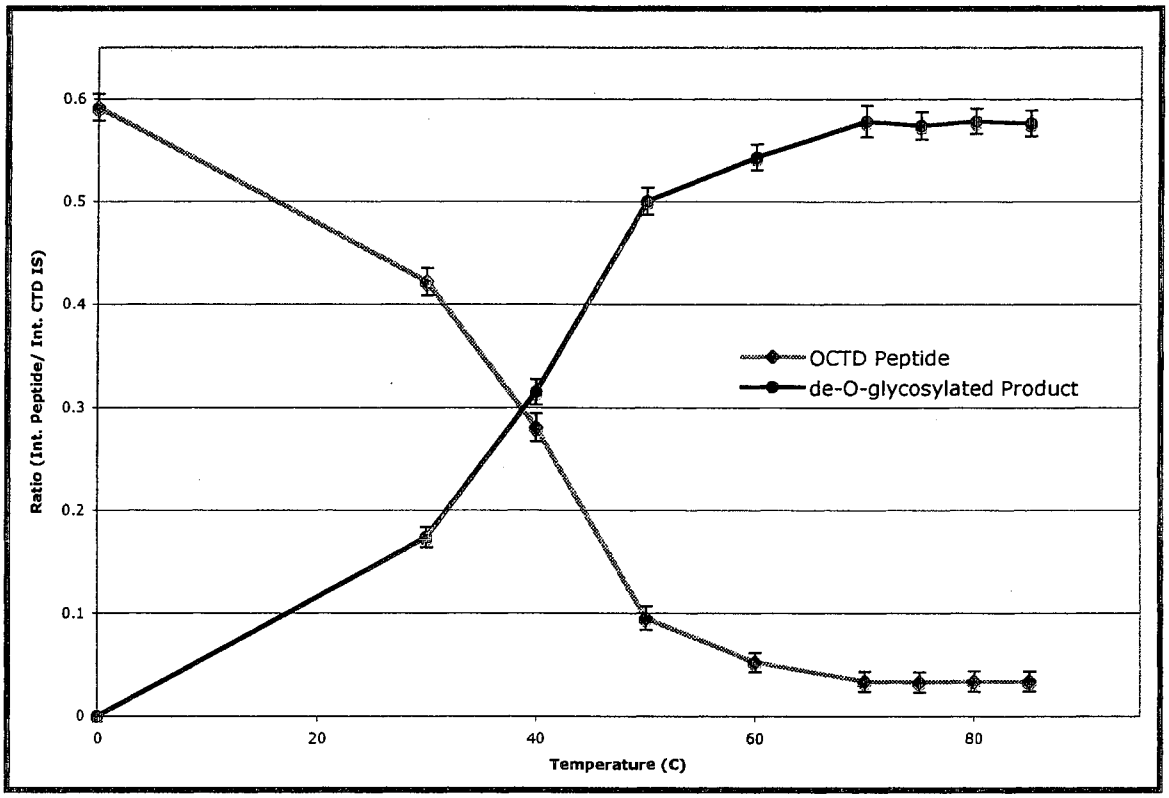


Figure 3.6 Microwave temperature optimization studies on the OCTD peptide relative to an internal standard (CTD). Maximum de-O-glycosylation was reached after a 70°C one hour reaction with 40% aqueous DMA. 5 trial average, error bars represent standard deviations.

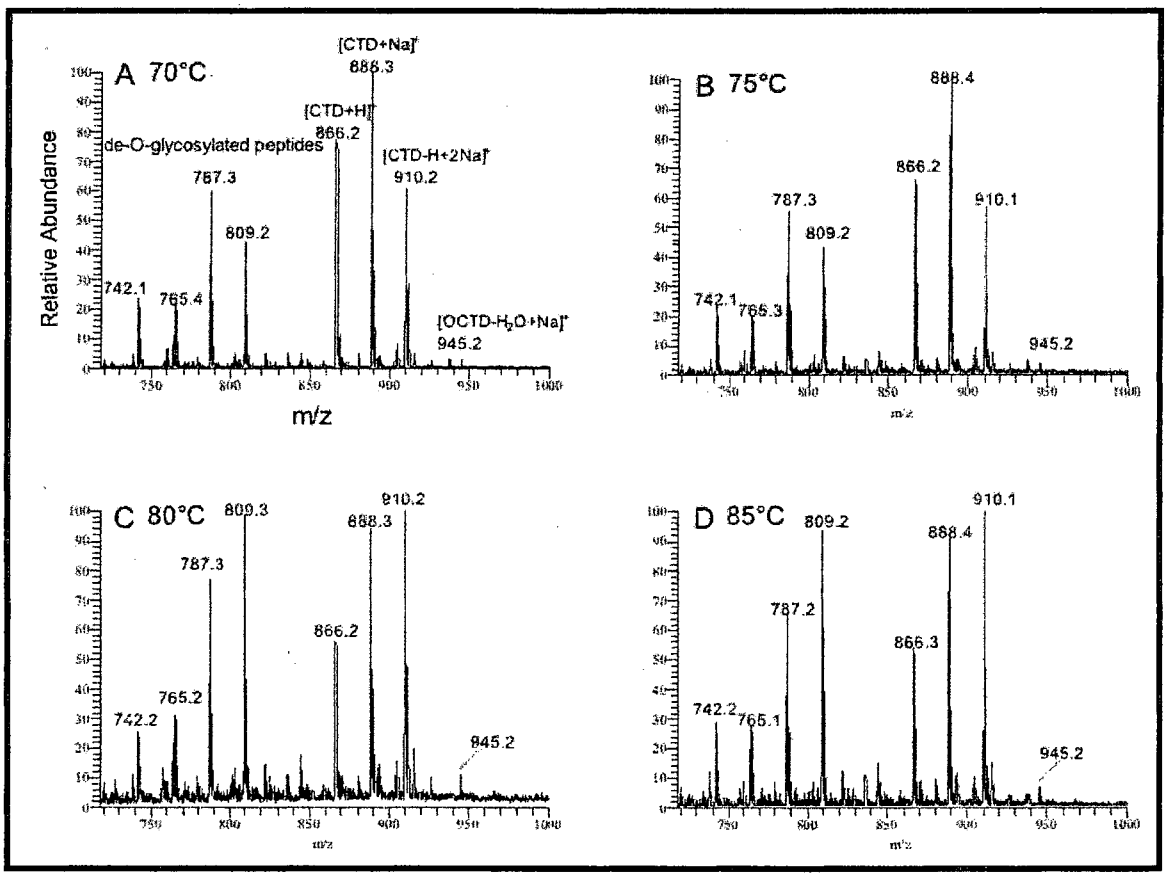


Figure 3.7:Effect of elevated reaction temperature on GlcNAc release from the OCTD peptide with CTD peptide as an internal standard. ESI MS of the reaction products after one hour in the microwave reactor at (A) 70°C; (B) 75°C; (C) 80°C; (D) 85°C. Only double bond or DMA peptide adducts are seen after de-O-glycosylation (Table 3.2)

Table 3.2: Structures of de-O-glycosylated peptide ions observed in Figure 3.7 after DMA-based microwave release. Each reaction product ion represents the loss of GlcNAc and formation of a DMA adduct or a double bond from the OCTD peptide

m/z	Structure
809	[OCTD-GlcNAc+DMA-H+2Na]+
787	[OCTD-GlcNAc+DMA+Na]+
765	[OCTD-GlcNAc+DMA+H]+
742	[OCTD-GlcNAc+Na]+

After one hour in the microwave at 70°C (Figure 3.7 A) only a small peak at 945 Da of the OGlcNac-CTD peptide remained, this ion remained at roughly the same abundance after the temperature was raised to 85°C. Therefore it was determined that the best reaction conditions were at 70°C with no addition of ammonium carbonate. Since an increase in the reaction temperature can lead to peptide and glycan hydrolysis, especially desialylation, the milder conditions were more favorable.

The next variable to be determined was reaction time. For these trials the microwave reactor was set at 70°C and reaction times from 0-120 min were evaluated. Ratios between the OCTD peptide and the CTD internal standard, and the de-O-glycosylated peptide product and CTD internal standard were calculated as described previously. Figure 3.8 shows the reaction time optimization graph, the release of the O-glycan leveled off at one hour, and approached but never reached 100%.

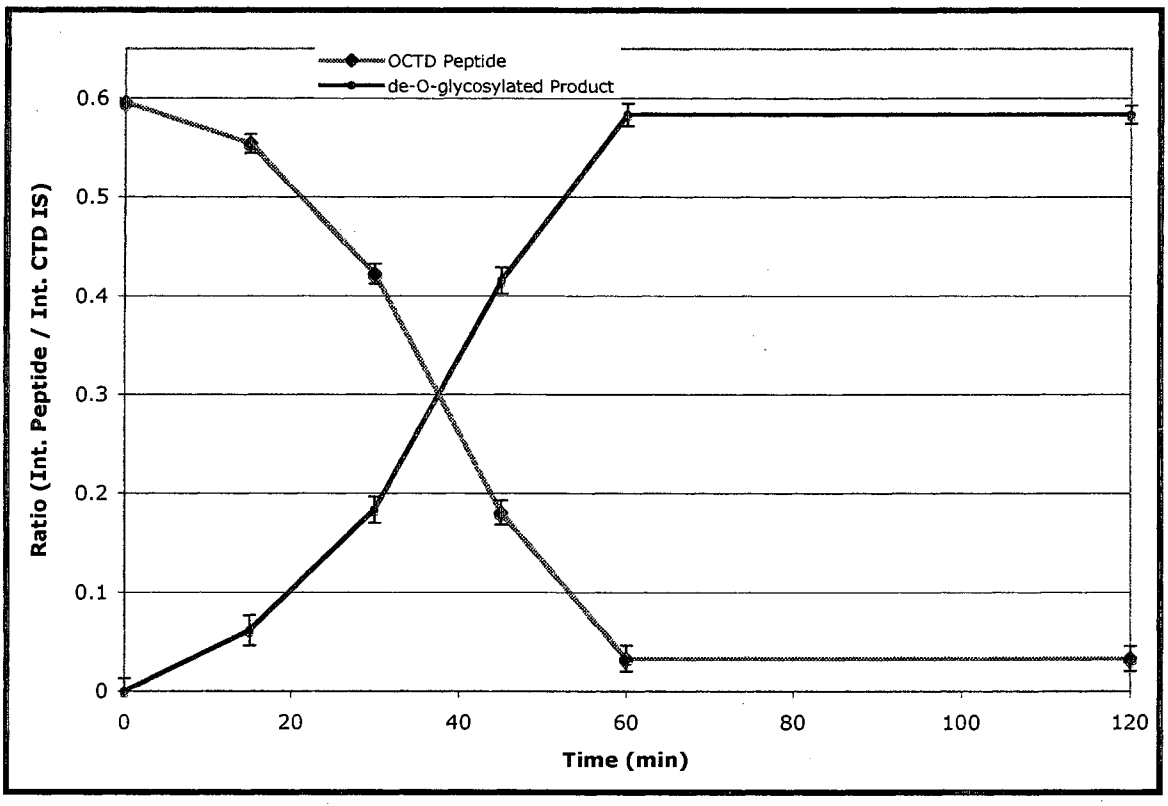


Figure 3.8: Microwave time optimization studies on the OCTD peptide relative to an internal standard (CTD). Maximum de-O-glycosylated product was obtained after a 70°C one hour reaction with 40% aqueous DMA. 5 trial average, error bars represent standard deviations.

The reason the reaction could not reach 100% release was due to the microwave reaction vessel, which monitors pressure during the reaction via an IntelliVent pressure control system as a safety control. In order to monitor the pressure the reaction vessel was sealed with a flexible septum, and this incomplete seal lead to the escape of the volatile DMA over time and is shown in a typical reaction monitor graph, Figure 3.9. Here the initial reaction pressure was 45 psi which decreased over time, to a final pressure of 15 psi, indicating

possible escape of the volatile organic base over time.

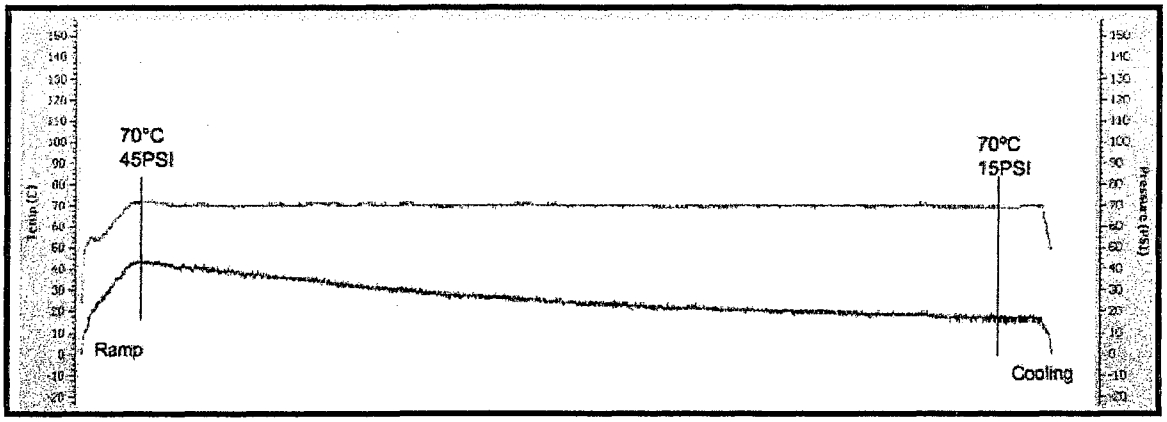


Figure 3.9 : Reaction parameter graph displaying temperature (°C) and pressure (psi) for a typical microwave-assisted aqueous DMA release

The release efficiency by microwave-assisted DMA de-O-glycosylation at 70 ℃ for1 hr was calculated to be 95% on average. To calculate the release efficiency Equation 3.1 was used. The ion abundances of all peaks corresponding to the glycopeptide (OCTD) were manually summed and were ratioed with all peaks corresponding to the the CTD internal standard. This ratio was then normalized to the zero minute unreacted standard (T=0).

$$\text{Release Efficiency} = 100 - \left[100 \times \left(\frac{\left(\frac{Int.OCTD}{Int.CTD} \right)_{T=X}}{\left(\frac{Int.OCTD}{IntCTD} \right)_{T=0}} \right) \right]$$

Equation 3.1: Calculation of the release efficiency for microwave-assisted de-O-glycosylation

To demonstrate the benefit of microwave radiation over conventional heating blocks, a sample of CTD and OCTD peptides was reacted with aqueous DMA in a heating block set at 70℃ for one hour. In Figure 3.10 it is clearly shown that significant levels of OCTD remained, represented by abundant ions at m/z 941.2, 963.3, and 985.2. Interestingly, the de-O-glycosylated product ions at m/z 783.3 and 742.2, correspond to a water adduct and double bond formation respectively. No DMA adducts were formed suggesting that the microwave radiation is necessary to efficiently create DMA adducts in such a short reaction time. In comparison of Figure 3.10 to Figure 3.7A it is evident that microwave irradiation is more efficient for de-O-glycosylation than conventional heating blocks, wherein complete de-O-glycosylation would take several hours.

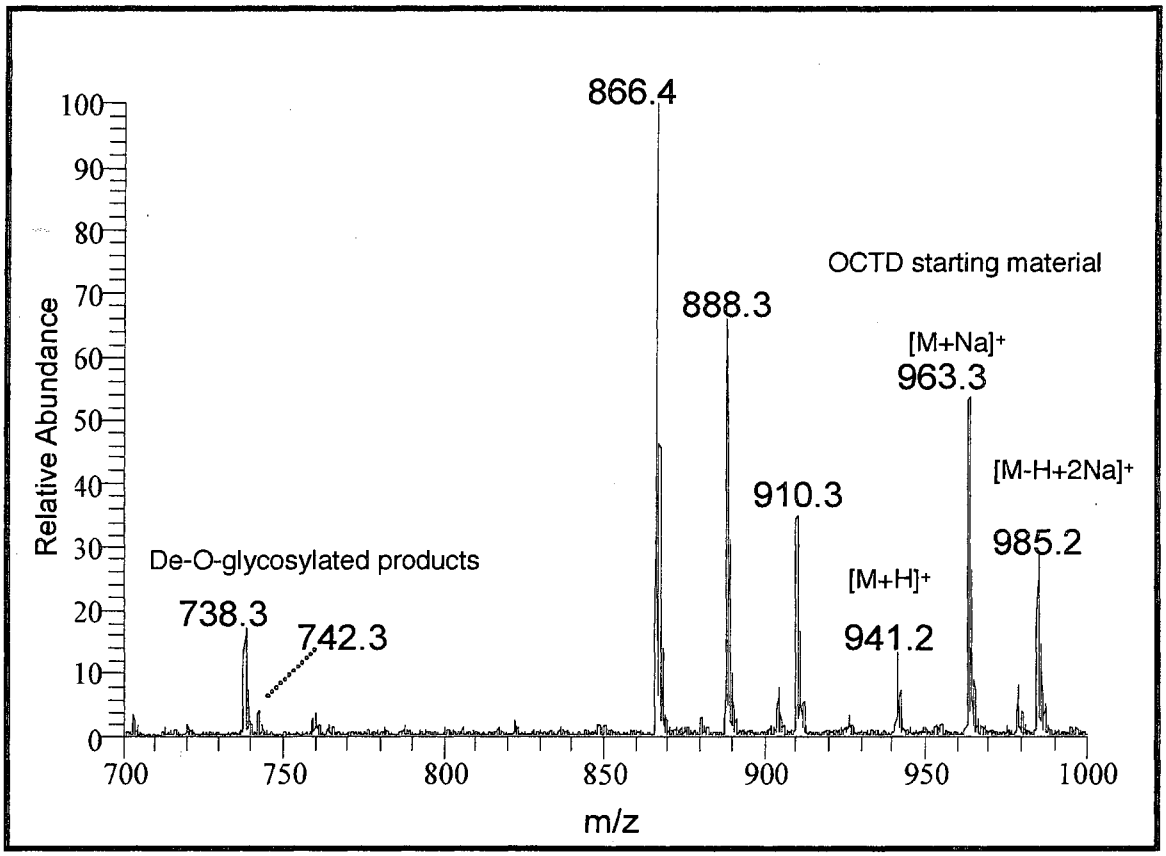


Figure 3.10: ESI MS of a sample of CTD and OCTD peptides subject to aqueous DMA in a pre-heated heating block for one hour at 70°C, release efficiency was calculated to be <10% on average

Utilization of Method for O-glycosylation Site Determination

Due to DMA adduction on the peptide after release, as shown in both Table 3.2 and Figure 3.7, the utilization of the method for glycosylation site determination was investigated. Figure 3.11 is an ESI MS profile of the reaction products after one hour reaction in the microwave. The most abundant de-O-glycosylated product ion, sodiated DMA adduct, m/z 787.3 [OCTD-GlcNAc+DMA +Na]$^+$ was chosen for O-glycosylation site determination by CID MS^n.

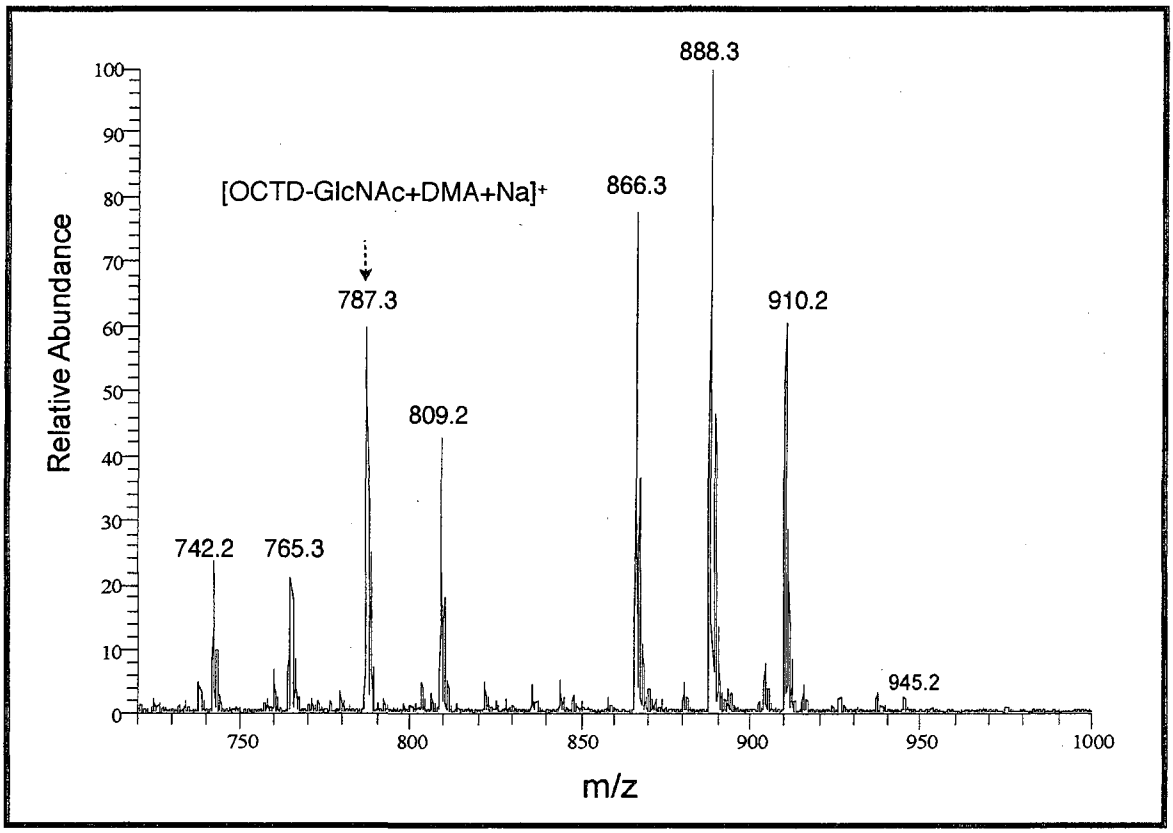


Figure 3.11: ESI MS profile of OCD and CTD after one hour 70°C reaction in the microwave with aqueous DMA. The ion at m/z 787.3, [OCTD-GlcNAc +DMA+Na]$^+$ was chosen for glycosylation site determination

An MS^2 spectrum of the ion at m/z 787.3 is shown in Figure 3.12, this spectrum showed that the most abundant CID ion was a loss of DMA and formation of double bond, m/z 742.4.

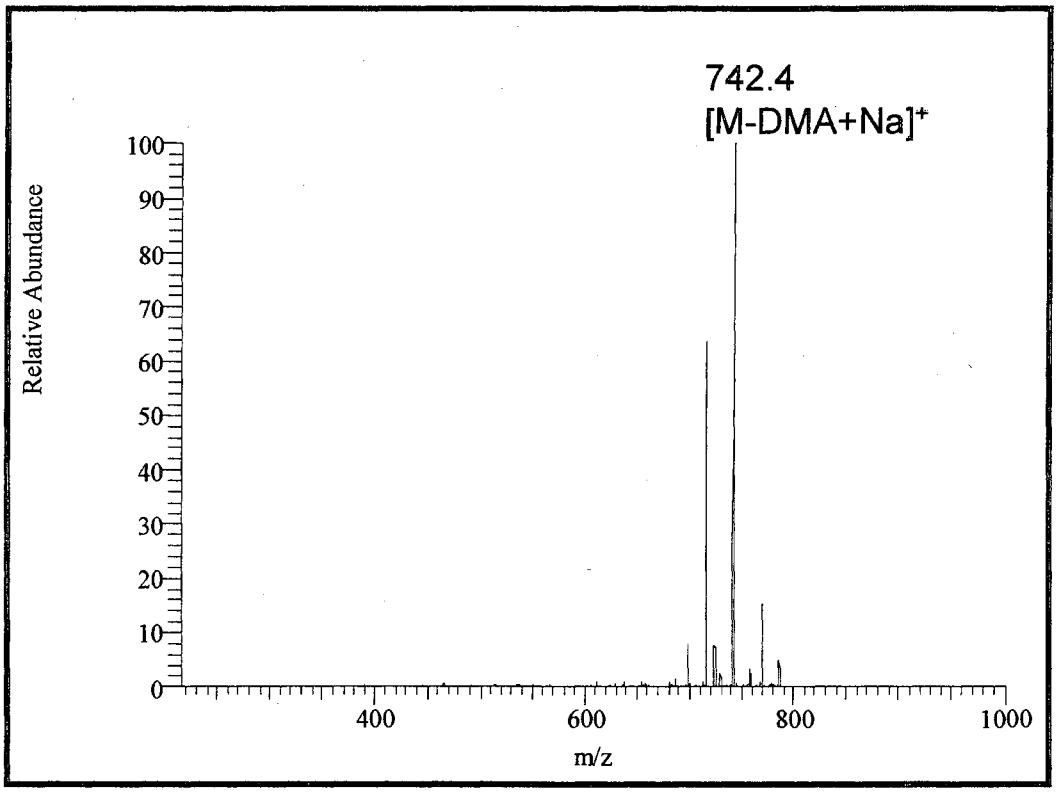


Figure 3.12: ESI MS^2 spectra of the ion at m/z 787.3. The abundant ion at m/z 742.4 was observed which corresponds to a neutral the loss of DMA.

Fragmentation of the 742.4 Da ion resulted in an MS^3 spectra containing abundant peptide backbone b- and y-type fragment ions. This is consistent with low energy CID reports of peptides, where positively charged amide bond fragmentation produced abundant b- and y- type fragment ions, containing the N and C terminus respectively. Losses of water and/or ammonia may also be present but residue side chain cleavages do not usually occur.[56]

This peptide sequence (YSPTSPS) has four possible glycosylation sites; serine^2, threonine^4, serine^5, and serine^7, as shown in Figure 3.13.

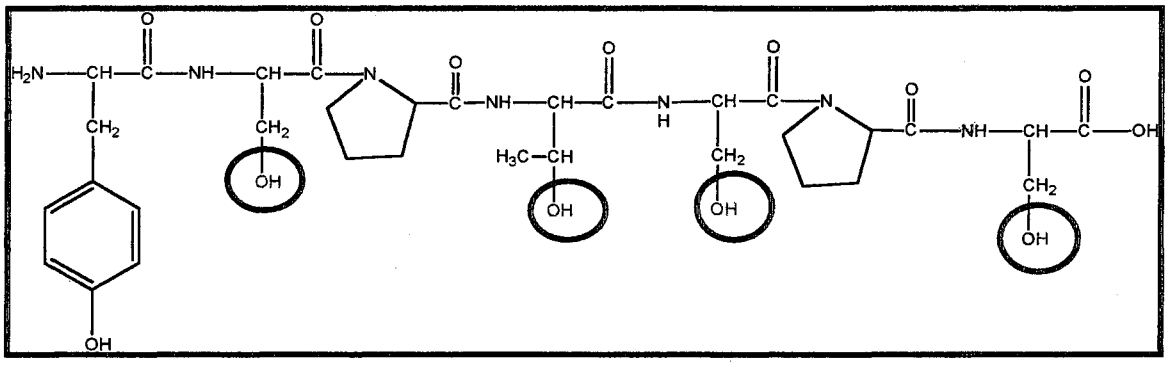
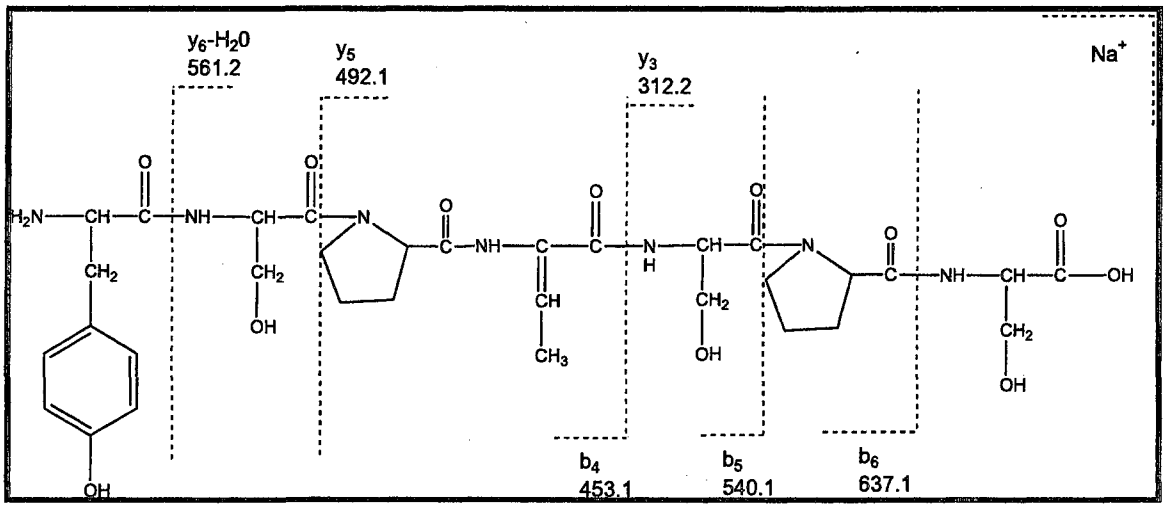


Figure 3.13: Structure of the CTD peptide used for glycosylation site determination. The four potential O-glycosylation sites are circled.

Analysis using MS^3 data, shown in Figure 3.14, showed that $serine^2$ was not the the glycosylation site, as the difference in mass from the y_6 ion and the y_5 ion, was 87 Da, which corresponds to a non-derivatized serine residue. $Serine^5$ and $serine^7$ were determined to not be the glycosylation site due to the presence of a y_3 ion which is at the native mass of the peptide. The y_5 ion and the b_4 ion were both 18 Da lower than they would appear in a non-glycosylated peptide. Of the two amino acids that were present in both the y_5 and b_4 ions, $threonine^4$ is the only residue capable of O-glycosylation. Therefore, it was concluded that Thr^4 was the glycosylation site of the OCTD peptide.



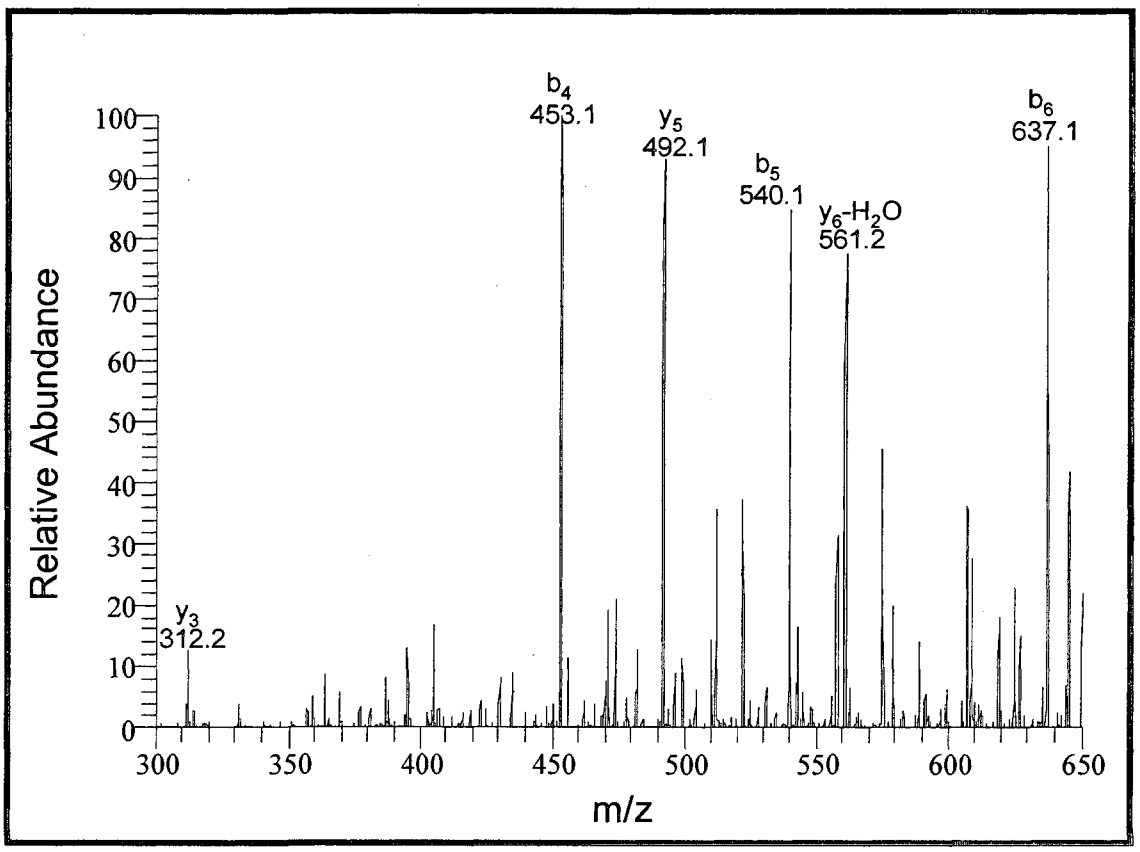


Figure 3.14: ESI MS^3 spectra demonstrating b- and y-type ions allowed for determination of the glycosylation site of the OGlcNacCTD peptide to be Thr^4

$DMAd_6$ Labeling of the Glycosylation Site

It was determined that one hour in the microwave reactor at 70°C with aqueous DMA allowed for efficient release of O-glycans. The reaction product contained a DMA adduct which was fragmented to reveal the site of O-glycosylation. Another issue to address was peptide selection from a complex mixture. In the above method development, synthetic peptides were utilized so the de-O-glycosylated peptide of interest was known. However, in a complex mixture of peptides and de-O-glycosylated peptides this is not the case. It may be possible to obtain MS^n data on each peptide in the fingerprint, however this process may be extremely time and sample consuming.

A method to aide in selection of former glycopeptides after de-O-glycosylation was demonstrated by use of a deuterium label. The reaction parameters were consistent with those determined earlier, however instead of utilizing aqueous DMA, a aqueous solution of a 50 mol% mixture of DMA and $DMAd_6$ was utilized.

Figure 3.15 shows the reaction products after the DMA/ $DMAd_6$ de-O-glycosylation and labeling. Peaks separated by 6 Da indicated the incorporation of the $DMAd_6$ and the release efficiency remained unchanged during this reaction. The method of 50% mixture of DMA and $DMAd_6$ allows for former O-glycosylated peptides to be visually selected from a complex mixture due to the unique doublet of peaks. It would be further useful at this point to attempt to de-salt or alter the sample mixture before ESI MS analysis in order to obtain one ion adduct. For example, acidifying the solution with acetic acid will cause an

increased abundance of H^+ ions. In this way the peptide fingerprint would be simplified, and O-glycopeptides would easily be selected and analyzed.

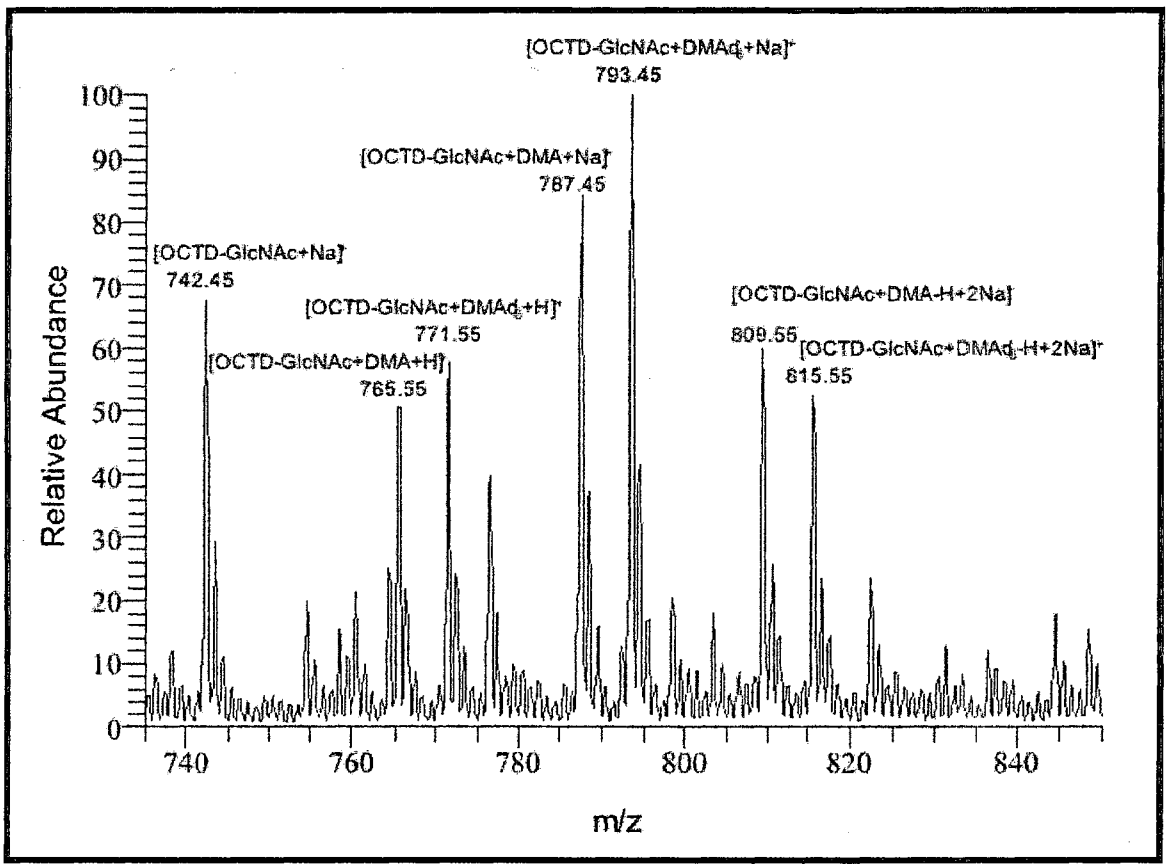


Figure 3.15 : ESI MS of the de-O-glycosylated peptides after release under DMA/ $DMAd_6$ conditions. Microwave release efficiency was not effected. Former O-glycopeptides may be selected based on doublet of peaks separated by 6 Da.

Effect of the Method on a Protein

With ideal time (one hour), temperature (70°C) and pH (~12.5) in hand the method was further examined on a standard protein, ribonuclease A. The MALDI MS spectra of reduced, alkylated, tryptic digested ribonuclease A is shown in Figure 3.16 A, the high mass peptides, which were previously outlined in Table

2.1 were observed and are labeled in the figure. After one hour in the microwave with aqueous DMA at 70°C, none of the tryptic peptides were detected in their native form. Abundant ions at m/z 1230.4 and 1252.4, which corresponded to a peptide bond cleavage of asparagine-proline were present, as shown in Figure 3.16B, other peptide cleavage products were also seen and are shown in Figure 3.18 and Table 3.5. Some of the tryptic peptides, while not observed in their original form, were observed as a DMA replacement on the cysteine residue, as determined by previous data by our group, Figure 3.17, yet other peptides were unable to be detected after the reaction, these peptides were cleaved and their products were too small to be effectively detected.

Each peptide containing cysteine was modified by DMA, this may be problematic for O-glycosylation site determination since the DMA modified cysteine and a DMA adduct on serine are identical, leading to false glycosylation site determination. The proposed 70°C for 60 min microwave reaction method may still be useful in glycosylation site determination if the O-glycan is on a stable peptide that does not containe cysteine, however for universal determination of O-glycosylation sites in unknown samples, these constraints are unfavorable.

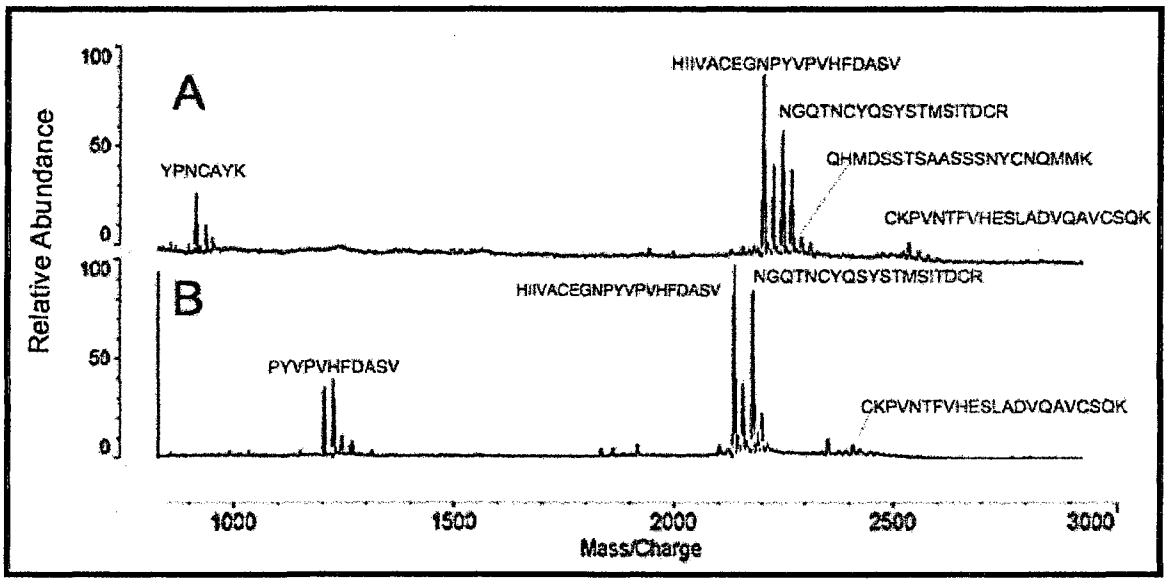


Figure 3.16: MALDI MS of Ribonuclease A tryptic peptides (A) before and (B) after microwave with aqueous DMA for one hour at 70°C

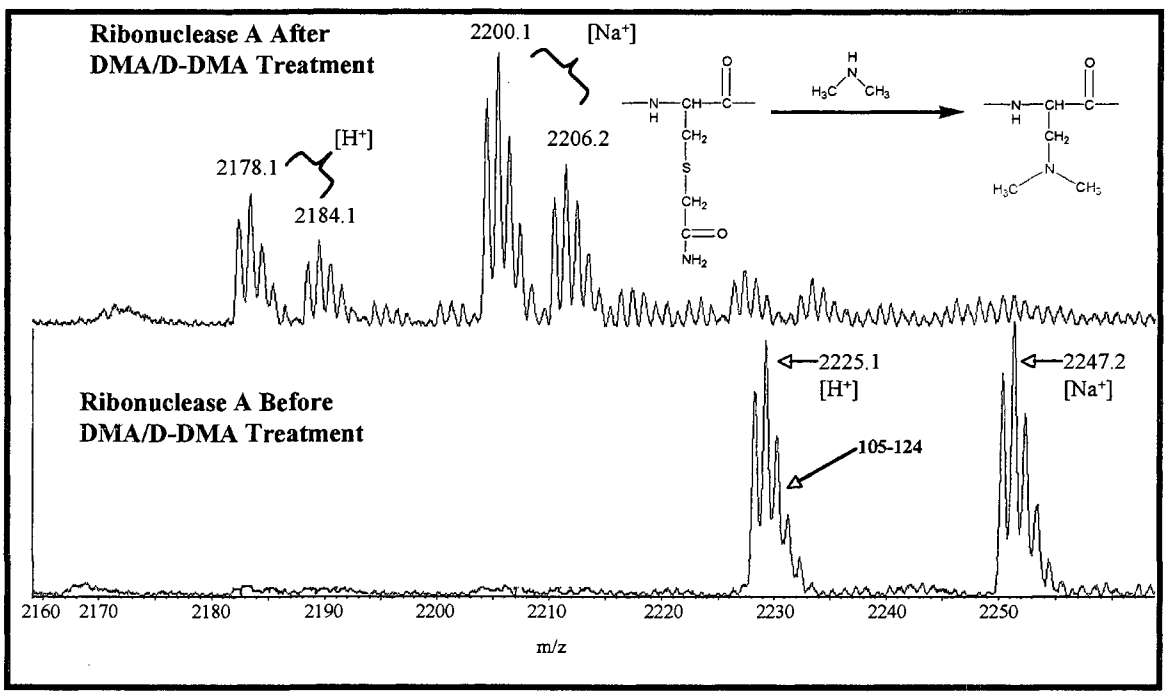


Figure 3.17: MALDI MS of DMA/ $DMAd_6$ treatment on ribonuclease A indicated that DMA may be incorporated onto the position of cysteine groups

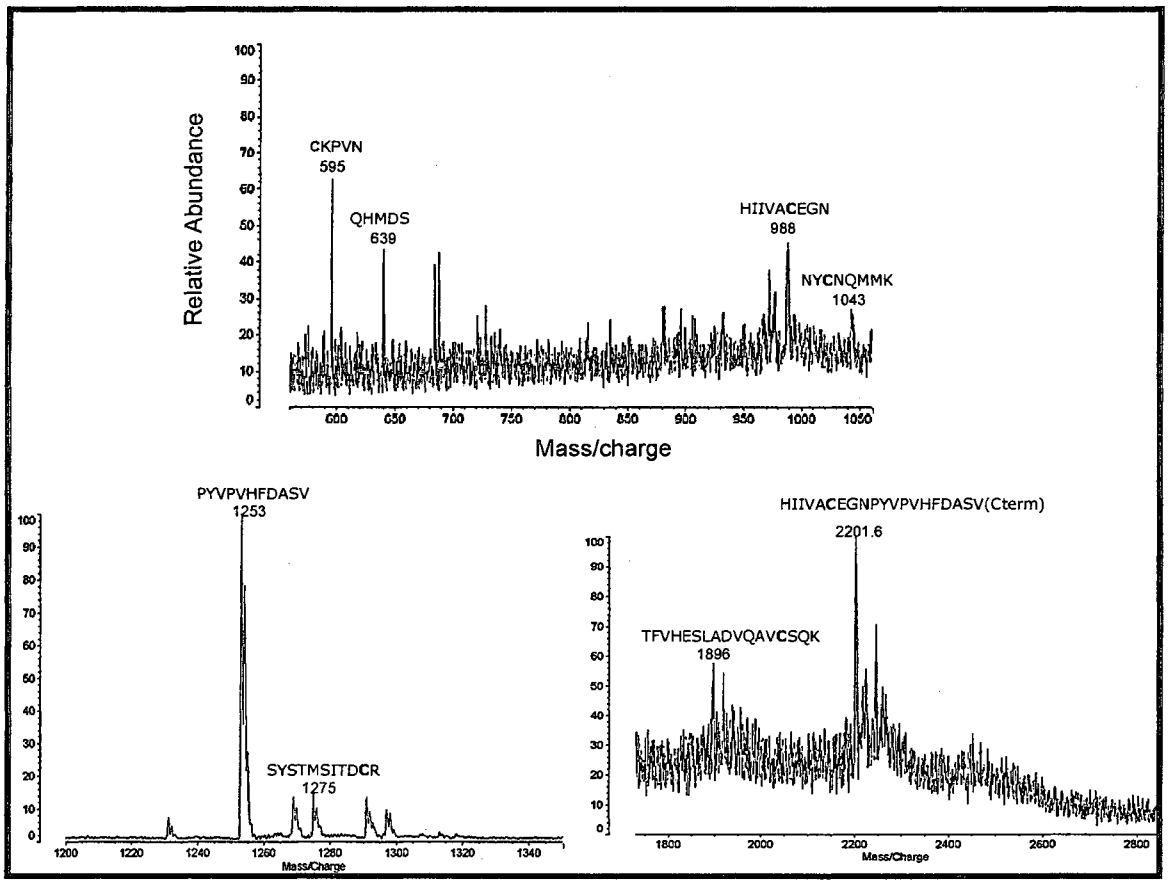


Figure 3.18: MALDI MS of the products from ribonuclease A tryptic peptides after 1 hr microwave reaction with aqueous DMA at 70°C.

To determine if the aqueous DMA or the microwave radiation caused the peptide hydrolysis, a sample of reduced, alkylated, and tryptic digested ribonuclease A is shown in Figure 3.19A, this sample was split in half. One half was heated with microwave radiation for one hour, and the other half was reacted with aqueous DMA at room temperature for 8 hours. The resulting MALDI MS spectra are shown in Figure 3.19 B and C. After one hour in the microwave reactor without DMA, the peptide fingerprint of ribonuclease A indicated no

change from the unreacted sample. However after reaction with DMA at room temperature for 8 hours, significantly different spectra were obtained. This data confirmed that any damage to the peptide backbone was due to the presence of DMA and not the microwave radiation or heat.

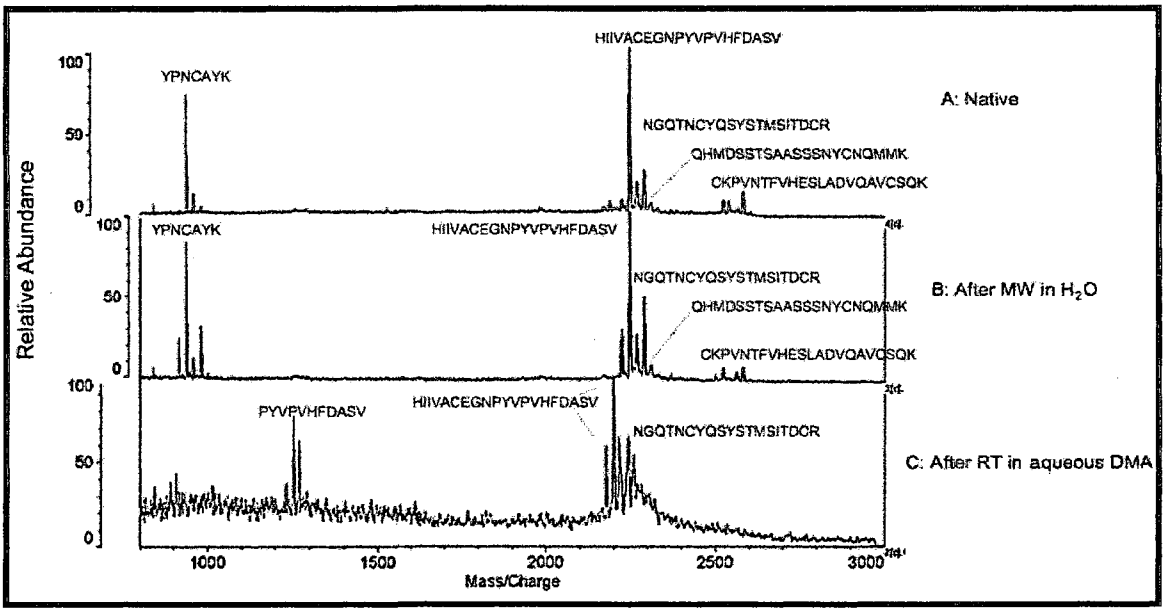


Figure 3.19: MALDI MS of ribonuclease A tryptic peptides. (A) Unreacted. (B) after one hour in the microwave with no DMA. (C) after 8 hours at room temp with aqueous DMA

Other reaction conditions, as shown in Table 3.3, were investigated to determine their effect on the peptide backbone and to find a balance between effective O-release and minimal peptide breakdown.

Table 3.3: Effect of various microwave reaction conditions on ribonuclease A tryptic peptides. All DMA-based conditions showed some peptide breakdown (X= peptide was observed in the spectrum)

Tryptic Peptides	Peptide Mass + Modifications	Tryptic Peptides	Zero Min	5Min DMA / 50°C	30Min DMA / 60°C	60Min DMA/ 70°C
CKPVNTFVHESLADVQAVCSQK	2516	X	X	X	X	X
QHMDSSTSAASSSNYCNQMMK	2364	X	X	X	X	
NGQTNCYQSYSTMSITDCR	2285	X	X	X	X	X
HIIVACEGNPYVPVHFDASV(Cterm)	2223	X	X	X	X	X
YPNCAYK	914	X	X	X	X	
TTQANK	661	X	X	X	X	
ETGSSK	607					
ETAAAK	589					
NVACK	590					
NLTK	474					
FER	450					
DR	289					
SR	261					
K	146					
ETGSSKYPNCAYK (missed cleavage)	1505	X	X	X	X	
SYSTMSITDCR	1275					X
TFVHESLADVQAVCSQK	1896					X
HIIVACEGN	988					X
QHMDS	639					X
PYVPVHFDASV	1231			X	X	X
NYCNQMMK	1043					X
CKPVN	595					X

Table 3.3 outlines the ribonuclease A tryptic peptides observed after a variety of reaction conditions. After only 5 minutes in the microwave at 50°C, the

asparagine-proline (N-P) peptide hydrolysis was evident. This labile cleavage was reported by Tarelli and Corran [35] by reaction of numerous standard peptides with aqueous ammonia, and appeared to be an unavoidable cleavage for any reaction condition tested. Figure 3.20 shows the tryptic peptides of ribonuclease A before and after the 30 minute, 60°C microwave reaction with aqueous DMA, under these conditions, only a small peak of peptide hydrolysis was evident and all peptides were identified in their native form.

Although, decreasing both reaction time and temperature from the optimal conditions, 70°C one hour, lead to less O-glycan released, as outlined in Figures 3.6 and 3.8, it is clear that the milder reaction conditions are needed when glycosylation site is of interest in unknown peptides. Furthermore, with shorter reaction times and reaction temperatures, there was no replacement of the cysteine residue with DMA, therefore, these conditions do not lead to false glycosylation site identification.

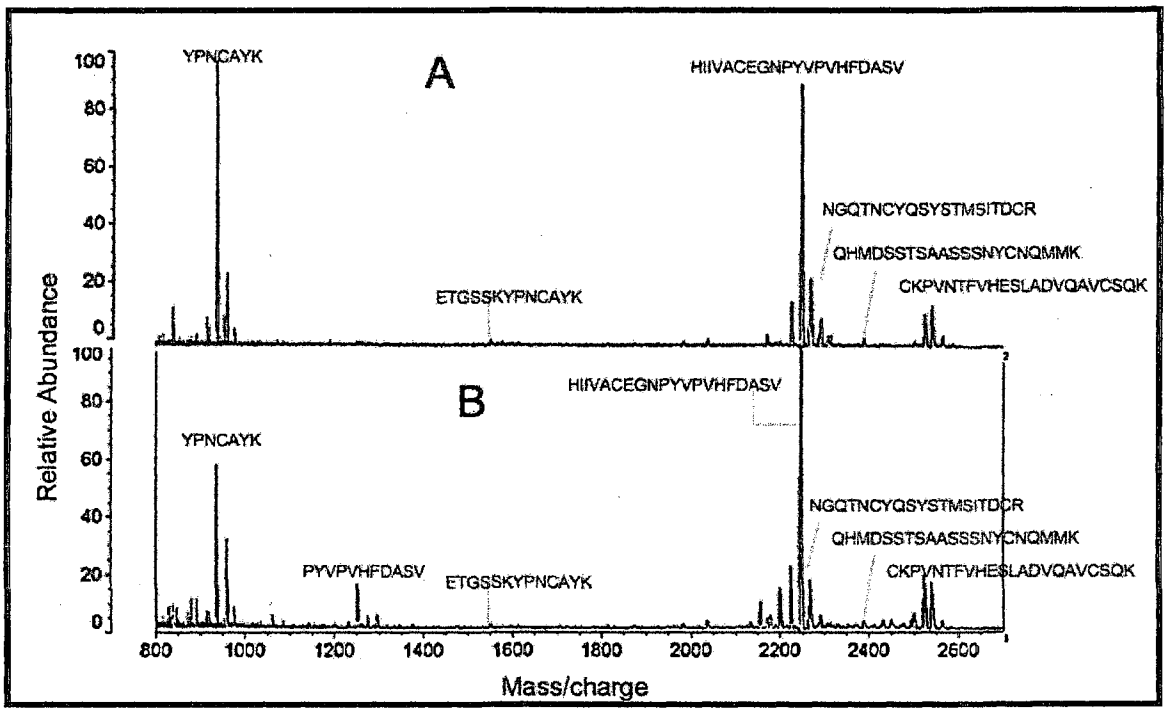


Figure 3.20: MALDI MS of the tryptic peptides of ribonuclease A (A) before and (B) after 30 min microwave radiation at 60°C in aqueous DMA

Figure 3.21 is a representative spectra of the OCTD/ CTD de-O-glycosylation reaction products after the 30 min 60℃ microwave reaction with aqueous DMA. The release efficiency was calculated using Equation 3.1 to be 75%.

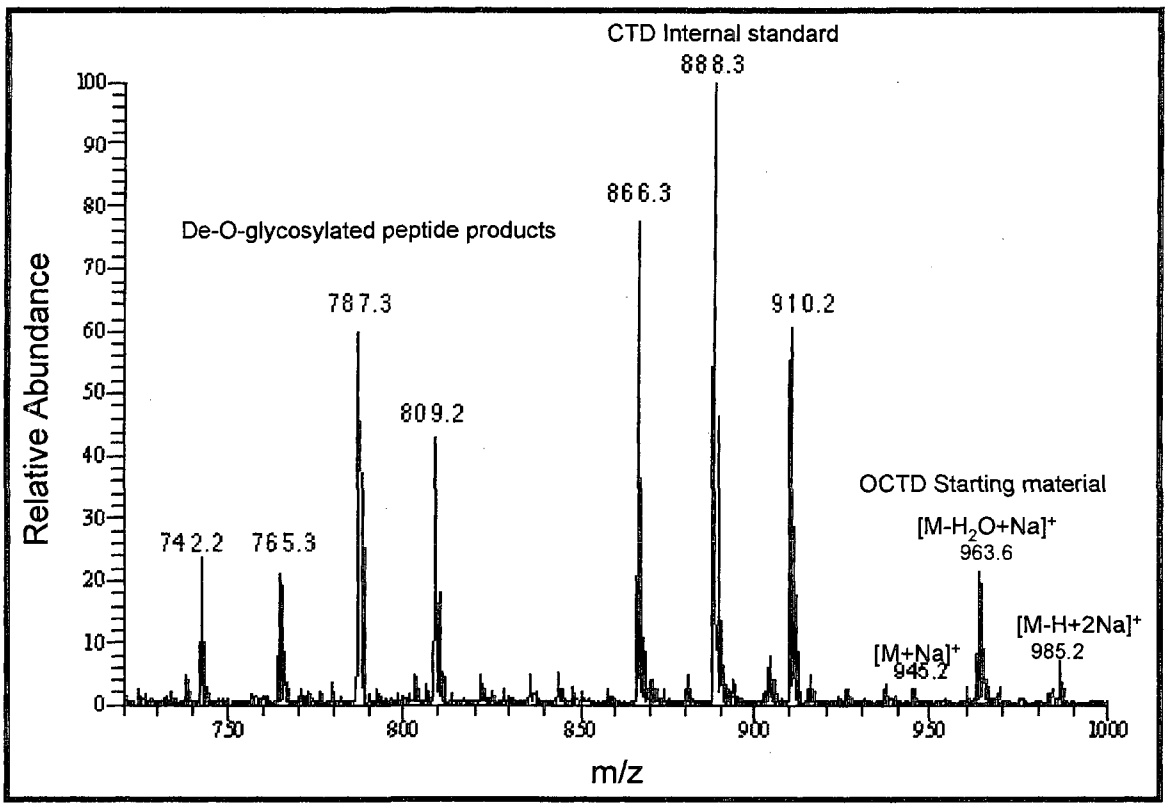


Figure 3.21: ESI MS of the OCTD de-O-glycosylation reaction products after a 30 minute microwave reaction at 60°C. On average, the release efficiency was 75%.

Summary

A microwave-assisted de-O-glycosylation method was developed by varying reaction time, temperature and pH. Ideal conditions for glycosylation site determination were: reaction of the glycopeptide with aqueous DMA/ $DMAd_6$ in the microwave reactor at 60°C for 30 min. Under these conditions, the release yield was 75% and minimal peptide degradation was observed.

The de-O-glycosylation results from varying the ammonium carbonate concentration showed inefficient release with saturated amounts. This result is

important because many researchers use ammonium carbonate to protect the released O-glycans from peeling, however its effect on the release efficiency had not been previously evaluated.

After the release reaction, glycopeptides were able to be selected because of ions separated by 6 Da in the mass spectrum, also this label did not interfere with the glycosylation site determination by ESI MS. Maximum de-O-glycosylation was obtained with microwave conditions of 70℃ for one hour. The results presented in this chapter clearly indicated that 95% release efficiency was obtained by this method. Since it has been shown that this method provided fast quantitative release, the method was further investigated for use in obtaining intact O-glycans for structural analysis by ESI MS^n, this will be discussed in Chapter IV.

CHAPTER IV

MICROWAVE-ASSISTED REDUCTIVE COMPLETE RELEASE FOR O-GLYCAN ANALYSIS

Introduction

If structural detail of the glycan is needed and labeling of the reducing end of the glycan is unnecessary, reductive release methods and mass spectrometry are used. *In situ* reductive methods have been developed in order to decrease O-glycan degradation by converting the free reducing end into an alditol. A reductive β-elimination method developed by D.M. Carlson in the 1960s utilized ionic strong base (NaOH) to release the O-linked glycans from the protein and a reducing agent ($NaBH_4$) to reduce and protect the glycans from degradation.[57] This so called "classical" release method remains the most common and reliable strategy so far. However, the addition of the reducing agent to prevent degradative peeling of the glycan (Scheme 2.2), has been shown to extensively degrade the peptide backbone.[58,59] Because of the significant peptide degradation, this method makes it difficult to quantify the peptide and therefore the extent of release has not been accurately determined. Additionally, this method involves a long reaction time and extensive post-reaction clean-up.

Although these problems are well known, the classical method is regarded as the most efficient and commonly utilized method to release O-glycans for subsequent analysis by MS.

It was shown in Chapter III that aqueous DMA and microwave heating for 60 min at 70℃ provided fast and efficient de-O-glycosylation with a release yield of 95%. Similarly, a reducing agent may be added to the reaction mixture in order to minimize the free O-glycans side reactions.

Goals of the Method

The goals of this method were first to evaluate the O-glycan yield under the release conditions developed in Chapter III. Next, the method was modified to incorporate a reducing agent ($NaBH_4$). A time course study was performed under the reductive conditions to confirm optimal reaction conditions. Finally, The established method was tested on two well known O-glycoproteins, bovine fetuin and porcine stomach mucin type III.

Experimental Methods

All microwave reactions were run on a CEM (Matthews, NC) Discover LabMate microwave equipped with an external infrared sensor for temperature feedback in closed vessel mode.

Materials

Porcine stomach mucin type III was purchased from Sigma (Saint Louis, MO). All other materials utilized in this chapter were described in Chapter II: Experimental Methods.

Non-reductive Microwave Release

An aqueous solution of 50 mg bovine fetuin and 50 µg β-cyclodextrin was prepared, and portions of this mixture were used for all analyses. An aliquot of the sample mixture containing 1 µg of cyclodextrin and 1 mg bovine fetuin was added to a 10 mL microwave reaction vessel and dissolved in 500 µL 40% aqueous DMA. The microwave was set to a reaction temperature of 70°C for one hour. Following the reaction, the reagents were evaporated under nitrogen gas then purified with a PGC column and permethylated as described in Chapter II: Experimental Methods.

Microwave Reductive Release

Two aqueous solutions, one of 50 mg bovine fetuin and one of 50 mg porcine stomach mucin type III, each with 50 µg β-cyclodextrin, were prepared. Portions of these mixtures were used for all analyses. An aliquot of the sample mixture containing 1 µg of cyclodextrin and 1 mg glycoprotein was added to a 10 mL pyrex reaction vessel along with 500 µL 40%DMA / 1 M $NaBH_4$. A small stir bar was added to equalize heating through the reaction, the reaction temperature was set at 70°C for 0 to 120 min.

The reaction was terminated by the addition of 1.5 mL acetic acid in an ice bath. Solvents were removed by evaporation in a SpeedVac. Borate esters were removed by repeat evaporations with 1% acetic acid in methanol under a stream of nitrogen gas. The sample was further purified with a PGC column and permethylated as described in Chapter II: Experimental Methods.

Traditional Carlson Release

An aliquot of the sample mixture containing 1 mg glycoprotein and 1 µg cyclodextrin was dissolved in 500 µL of aqueous 50 mM NaOH/ 1 M $NaBH_4$ in a glass test tube. The mixture was heated to 50°C or 70°C and reacted for 16 or 24 hrs with occasional venting of hydrogen gas. The reaction was terminated by the addition of 1.5 mL acetic acid in an ice bath. Solvents were removed by evaporation in a SpeedVac. Borate esters were removed by repeat evaporations with 1% acetic acid in methanol under a stream of nitrogen gas. The sample was further purified with a PGC column, and permethylated as described in Chapter II: Experimental Methods.

MS Methods

Electrospray ionization mass spectrometry (ESI MS) and Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) were performed as described in Chapter II: Experimental Methods.

Results and Discussion

Comparison of DMA Based Microwave-Assisted O-glycan release to Classical Release

In order to apply the method developed in Chapter III to O-glycan analysis,

two experiments on bovine fetuin O-glycan release were performed. A sample was reacted in aqueous DMA with microwave conditions of one hour, 70°C, another sample was subject to classical NaOH/ $NaBH_4$ release in a heating block at 50°C for 16 hrs. After O-glycan release and purification, the O-glycans were permethylated for accurate comparison. The results of the two experiments are presented in Figure 4.1.

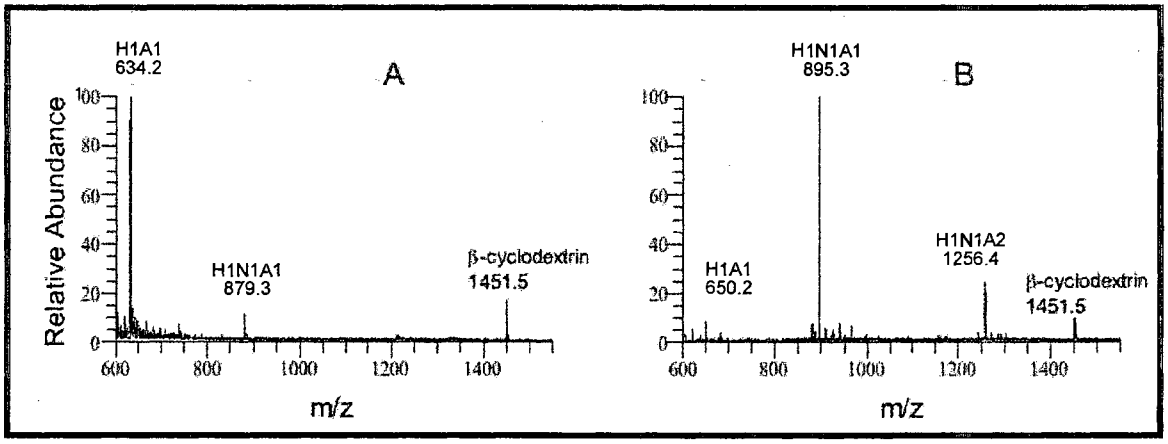


Figure 4.1: ESI MS comparison of two O-glycan release methods on bovine fetuin relative to β-cyclodextrin internal standard. (A) aqueous DMA, microwave heating for one hour 70°C (B) NaOH / $NaBH_4$ 16 hr 50°C in a heating block.

As expected from the results presented in Chapter III, the non-reductive conditions were effective in releasing O-glycans; Figure 4.1A shows an ion corresponding to O-1 (H1N1A1) O-glycan. However, significant peeling occurred, which can be observed in the same spectrum by P-1 (H1A1). Less intact O-glycan yield was evident when compared to the classical Carlson (reductive) release of the same sample, as shown in Figure 4.1B. Less yield was attributed to increased peeling due to the presence of non-reduced glycans in basic solution.

Re-evaluation of Method Conditions After Introduction of a Reducing Agent

As discussed in Chapter III, an aqueous DMA solution was able to release O-glycans efficiently at reaction conditions of 70°C for one hour under microwave irradiation (Figure 3.11). However, significant glycan peeling products were observed (Figure 4.1A). Therefore, simultaneous introduction of a reducing agent, $NaBH_4$, was applicable to prevent the peeling reaction.

The microwave reaction time under aqueous DMA/ $NaBH_4$ was re-optimized on bovine fetuin. A series of microwave heating times were evaluated from 0 to 120 min while reaction temperature remained constant at 70°C, the results are summarized in Figure 4.2. This graph is of the relative ion abundance ratio for bovine fetuin O-1 (H1N1A1) and O-2 (H1N1A2) glycans relative to the β-cyclodextrin internal standard. It was found that a one hour reaction produced the maximum yield for both O-glycan compositions, and a prolonged reaction time (120 min) did not increase the yield. Therefore, it was confirmed that a 1 hour reaction in the microwave reactor at 70°C is optimal to obtain the maximum release using the DMA/ $NaBH_4$ mixture.

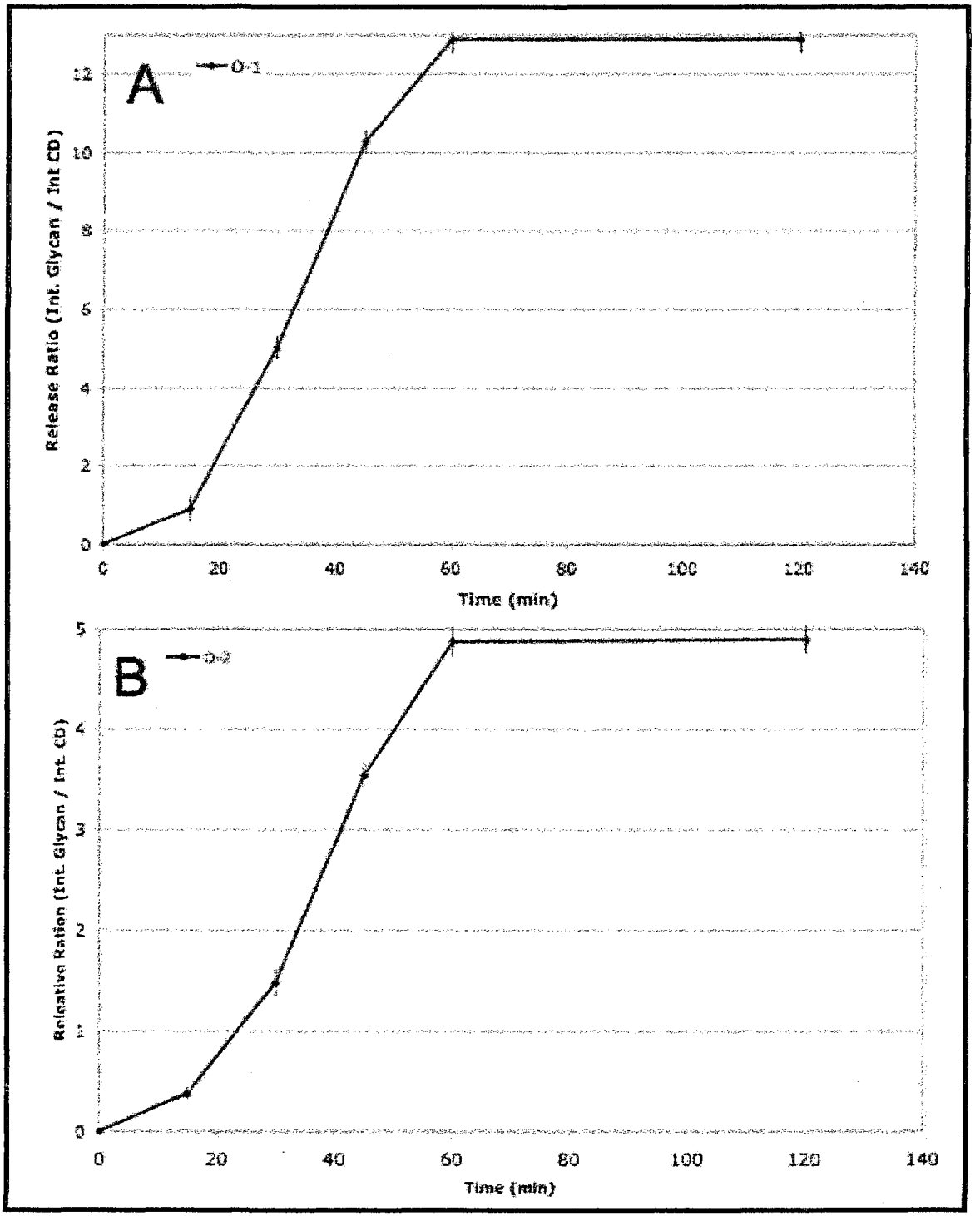


Figure 4.2: Microwave time optimization studies on O-glycans released from bovine fetuin relative to β-cyclodextrin internal standard showed maximum release after one hour reaction time at 70°C with aqueous DMA/ NaBH4. 5 trial average. Error bars reported are standard deviations. (A) O-1 glycan; (B) O-2 glycan

Microwave Assisted Reductive O-glycan release: Comparison to Classical Methods: Bovine Fetuin

Structural characterization of bovine fetuin O-Glycans was discussed in Chapter II. *O*-glycans were released from bovine fetuin using both the microwave-assisted DMA/ $NaBH_4$ (70°C and 1 hr irradiation) and classical heating block (50°C 16hr) reaction conditions. β-cyclodextrin was included in the reaction mixture as the internal standard to measure the relative release efficiency. One example ESI MS spectrum of each experiment is shown in Figure 4.3. Figure 4.3A is the spectrum from the microwave conditions and Figure 4.3B is from the heating block conditions. The peak pattern in the classical heating trial was similar to the microwave method, except a relatively high abundance of internal standard ion was observed in the classical method. A higher relative abundance of internal standard was indicative of a lower abundance of O-glycans, therefore the microwave-assisted method provided a higher yield of O-glycans.

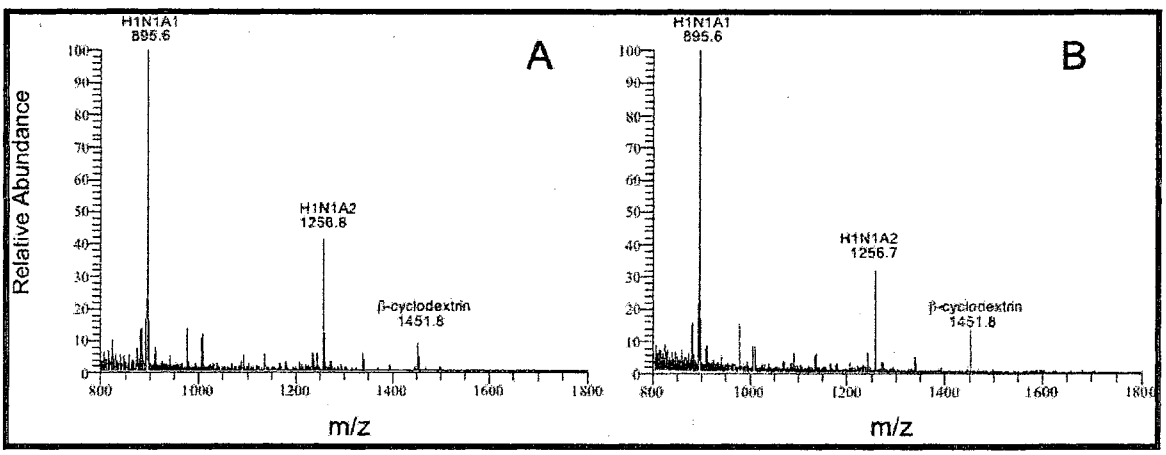


Figure 4.3: ESI MS comparison of O-glycan release strategies on bovine fetuin. A: Microwave DMA/ $NaBH_4$, 1hr 70°C. B: Classical NaOH/$NaBH_4$ 16 hrs 50°C (H:Hexose, N: HexNAc, A: Sialic acid, Internal standard was β-cyclodextrin)

The relative reaction yield of each O-glycan composition was calculated by finding the ratio of the ion abundance for each O-glycan relative to the ion abundance for the internal standard (β-cyclodextrin). Once the peak intensity ratio was determined for each trial of each method, the relative reaction yield was calculated by comparing the intensity ratios. This data is summarized in Table 4.1. The yield from the microwave-assisted method was, on average, 1.71 times greater than classical method for the O-1 O-glycan (H1N1A1, m/z 895), and for the O-2 O-glycan (H1N1A2, m/z 1256) yield was, on average, 1.93 times greater. These results clearly indicate that the classical NaOH/ $NaBH_4$ method cannot release O-glycans 100% efficiently, the microwave-assisted method provides a better yield.

Table 4.1: Comparison of reaction yields of classical and microwave-assisted de-O-glycosylation on bovine fetuin O-glycans relative to β-cyclodextrin internal standard. 5 trial average

m/z	Peak intensity Classical method relative to cyclodextrin internal standard	Peak intensity Microwave method relative to cyclodextrin internal standard	Reaction Yield Microwave/ Classical
895 (O1)	7.54	12.9	1.71 +/- 0.08
1256 (O2)	2.58	4.99	1.93 +/- 0.09

Microwave-Assisted Reductive O-glycan release: Comparison to Classical Methods: Porcine Stomach Mucin, Type III

In the glycomic field, methodology is typically tested on bovine fetuin, as was done in this chapter, since it is a well characterized glycoprotein with simple, abundant O-glycans. Porcine stomach mucin type III is a heavily glycosylated complex glycoprotein, this mucin was utilized to further show the capabilities of the new microwave-assisted reductive method. Several replicate experiments of O-glycan release of porcine stomach mucin by both the microwave-assisted DMA/ $NaBH_4$ and classical heating block NaOH/ $NaBH_4$ release were performed. Two representative spectra from these experiments are shown in Figure 4.4

Figure 4.4A and B are MALDI mass spectra of the released O-glycans from the microwave-assisted method and the classical heating method respectively. Both spectra contained the same glycan compositions, however the abundance of each O-glycan signal relative to β-cyclodextrin differed. The relative abundance of the β-cyclodextrin signal in Figure 4.4A was smaller than in Figure 4.4B indicating that a higher yield of O-glycans was obtained by the microwave-assisted method. As in the previous section, the relative ion abundance of each O-glycan was ratioed to the relative ion abundance of β-cyclodextrin for each spectrum and are listed in Table 4.3. Microwave-assisted O-glycan release produced a higher yield for each composition than the classical method, further demonstrating that the microwave-assisted method will release O-glycans more efficiently, and the classical method is not an accurate method for quantification, even though it has previously been considered as the most complete method.

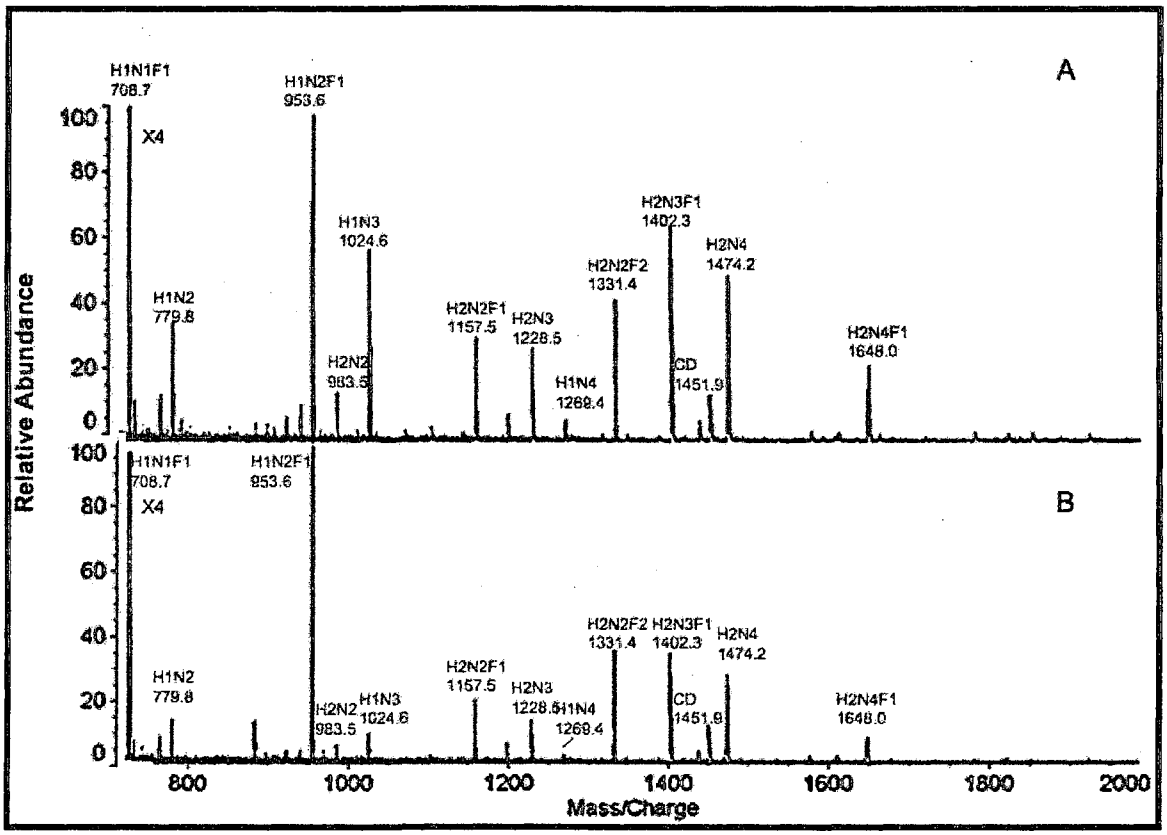


Figure 4.4: MALDI MS comparison of O-glycan release strategies on porcine stomach mucin type III. (A) Microwave DMA/NaBH4, 1hr 70°C (B) Classical heating block NaOH/$NaBH_4$, 16 hr 50°C (H:Hexose, N: HexNAc, F: Fucose, CD: β-cyclodextrin internal standard)

Table 4.2: Comparison of reaction yields of classical and microwave-assisted de-O-glycosylation on porcine stomach mucin type III O-glycans relative to β-cyclodextrin internal standard, 5 trial average.

O-glycan composition	Peak intensity Classical method relative to cyclodextrin internal standard	Peak intensity Microwave method relative to cyclodextrin internal standard	Reaction Yield Microwave/ Classical
H1N1F1	3.05	3.95	1.30 +/- 0.05
H2N2	1.05	1.33	1.26 +/-0.03
H1N2F1	1.27	1.67	1.31 +/- 0.06
H2N2	0.55	0.71	1.29 +/- 0.04
H1N3	1.21	1.55	1.28 +/- 0.05
H2N2F1	0.82	1.01	1.24 +/- 0.05
H2N3	0.82	1.00	1.22 +/- 0.04
H1N4	0.30	0.37	1.22 +/-0.04
H2N2F2	1.04	1.22	1.17 +/- 0.06
H2N3F1	1.84	1.94	1.06 +/- 0.04
H2N4	0.92	1.09	1.18 +/- 0.05
H2N4F1	0.42	0.47	1.13 +/- 0.04

Finally, the sensitivity of the method was evaluated on a 10 µg sample of porcine stomach mucin. The sample was not permethylated since significant sample loss may occur during the repeat liquid-liquid extractions following the permethylation reaction, also no sialic acid containing glycan were observed in Figure 4.4. The obtained O-glycan profile is shown in Figure 4.5. All O-glycan compositions observed in Figure 4.4 were seen in this profile, which

demonstrated that this method is applicable when limited sample quantities are available.

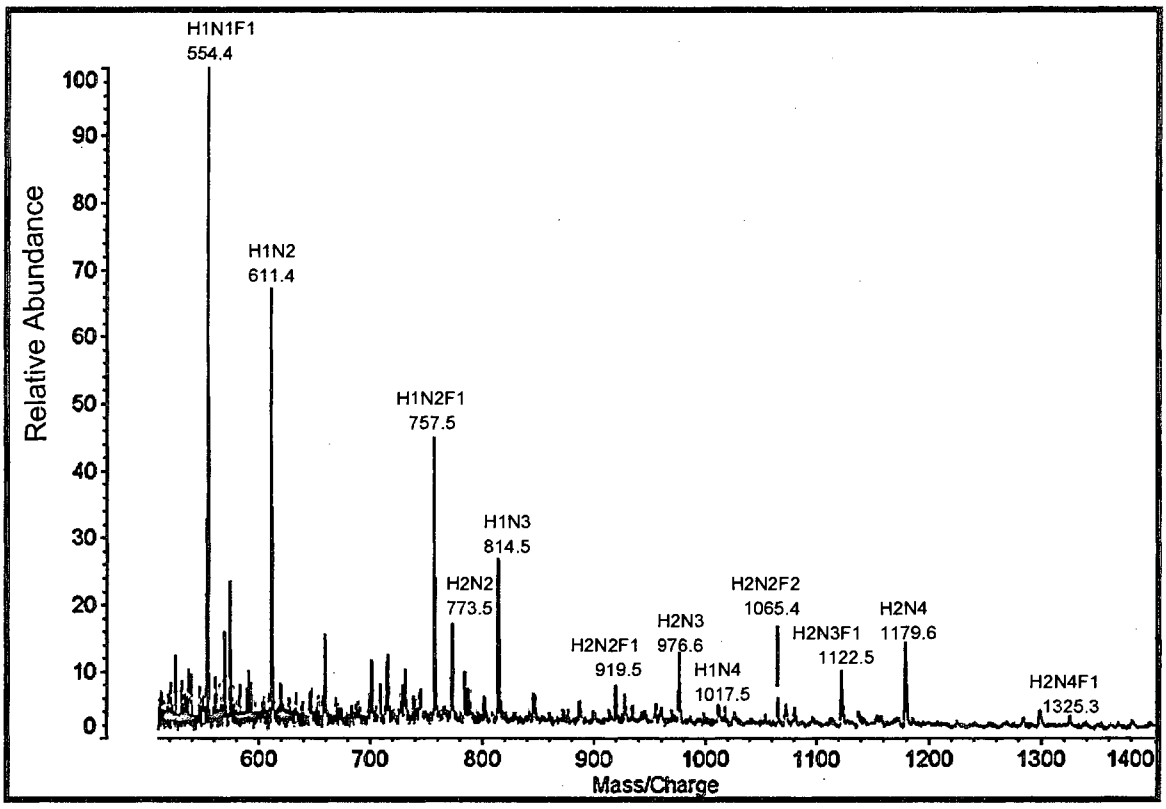


Figure 4.5: MALDI MS Profile of O-glycans released from 10 µg of porcine stomach mucin type III by microwave-assisted DMA/ $NaBH_4$ 1 hr 70°C. (H=hexose, N=HexNAc, F= Fucose)

This microwave-assisted aqueous DMA/ $NaBH_4$ method provided a simple, fast, and efficient method for O-glycan release with increased yields of O-glycans relative to the conventional method. Although the classical NaOH/$NaBH_4$ method has not been carefully evaluated, it is most common to utilize a reaction temperature of 50°C and a reaction time of 16 hrs. As shown, this will not provide complete release.

Evaluation of Classical Heating at Different Reaction Conditions. Comparison to Microwave Heating.

In the previous sections it was shown that O-glycan release by the microwave-assisted reductive method had an increased yield of O-glycans compared to the classical heating block based method. However, classical release has not been carefully optimized. O-glycan release under NaOH/ $NaBH_4$ in a heating-block was evaluated for a longer reaction time (24 hrs at 50°C), and higher temperature (16 hrs at 70°C). A representative spectrum for each condition on de-O-glycosylation of bovine fetuin is shown in Figure 4.6.

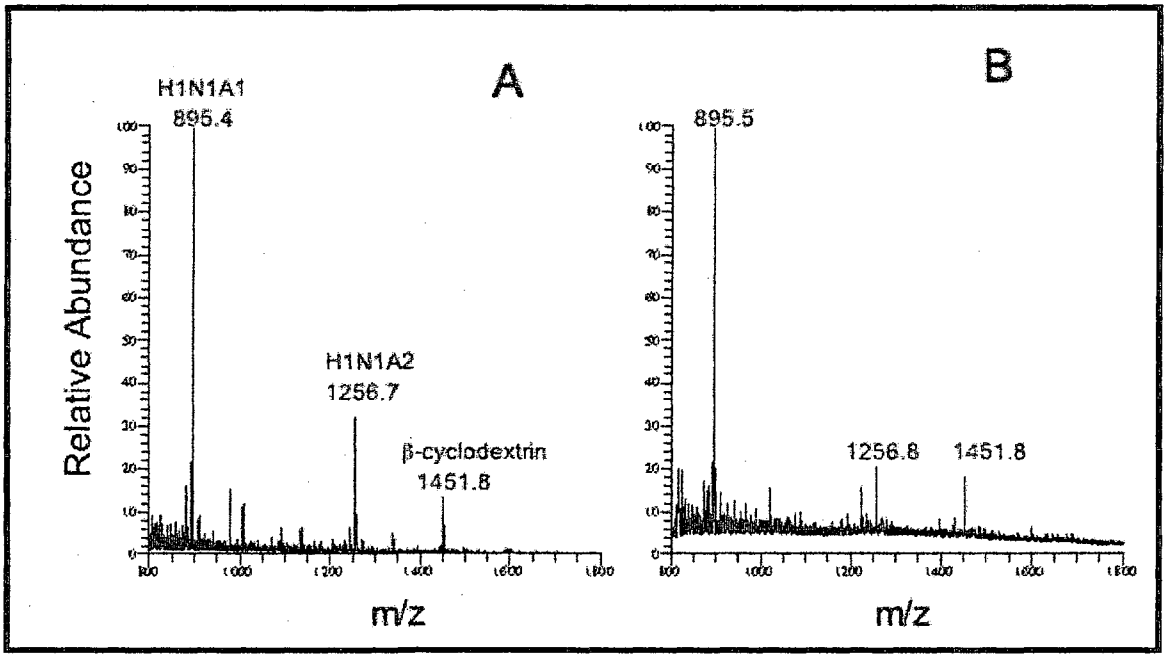


Figure 4.6: Example ESI MS spectra for the comparison of variations in the classical method. (A) Classical NaOH/ $NaBH_4$ release at 50°C for 24 hr (B) Classical release with increased heating to 70°C for 16 hr

The relative reaction yield was calculated as explained above. Reported in Table 4.3 are the relative reaction yield comparisons of the new microwave-assisted method to the classical method, and comparisons of the classical method to the time and temperature extended conditions for the O-glycans released from bovine fetuin. In comparison to the classical 16 hr 50℃ release, the 24 hr 50℃ conditions provided the same yield of glycans O-1 and O-2 and major peeling product P-1. In comparison of the classical 16 hr 50℃ to the 16 hr 70℃ reaction conditions, the reaction yield for both O-1 and O-2 glycans was larger under the 50℃ conditions. The decrease in intact O-glycan yield was due to a higher abundance of degradative peeling in the 70℃ reaction conditions, this may be seen by a higher reaction yield of P-1 (H1A1, major peeling product) under these conditions. Neither extending the reaction time nor raising the reaction temperature improved O-glycan yield compared to the microwave-assisted method. Therefore it was concluded that classical heating-block based methods do not provide complete release of O-glycans, microwave heating is essential.

Table 4.3: Comparison of O-glycan release methods at various conditions on bovine fetuin O-glycans relative to β-cyclodextrin internal standard. 5 trial average Microwave method provided higher yield in all cases

m/z	Reaction Yield Microwave 60 min 70°C / Classical 16 hr 50°C	Reaction Yield Classical 16 hr 50°C / Classical 24 hr 50°C	Reaction Yield Classical 16 hr 50°C / Classical 16 hr 70°C
895 (O1)	1.71 +/- 0.08	1.01 +/- 0.07	1.37 +/- 0.08
1256(O2)	1.93 +/- 0.11	1.07 +/- 0.08	2.03 +/- 0.08
634 (P1)	0.63 +/- 0.04	0.96 +/- 0.06	0.67 +/- 0.04

Summary

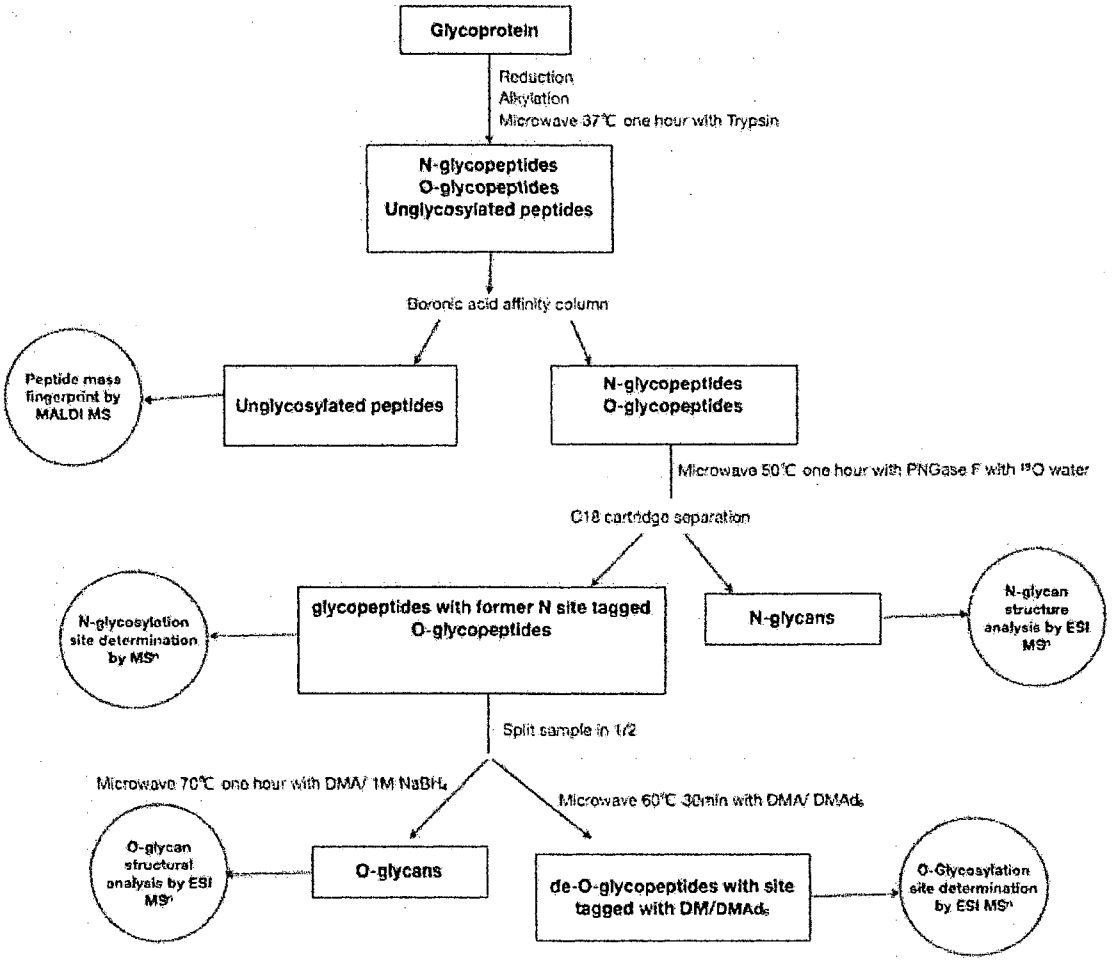
The optimal reaction conditions for reductive de-O-glycosylation were determined to be an aqueous solution of 40% DMA/ 1 M $NaBH_4$, in the microwave reactor for one hour at 70°C. Utilizing this method, release yields were shown to be higher than classical β-elimination for both glycoproteins tested, while reaction time was significantly shorter. Increasing the reaction time or temperature of the classical release provided no further increase in yield. Therefore, the method which provided the highest yields of O-glycans was the new microwave method.

CHAPTER V

TOWARDS MICROWAVE-ASSISTED SEQUENTIAL N- AND O-RELEASE

Introduction

It was the initial goal of this thesis work to develop a de-O-glycosylation method that could, along with established de-N-glycosylation methods, provide for sequential N- and O-glycan release. Although a single method which could provide released O-glycans for analysis as well as O-glycosylation site determination was unable to be developed, two individual methods were. Therefore, it is suggested that the sample be split in half prior to O-glycan studies, the O-glycosylation site may be determined by the method developed in chapter III (microwave with 40% aqueous DMA for 30 min at 60°C) and the O-glycan may be release and studied by MS methods by the method developed in chapter IV (microwave with 40% DMA/ 1 M $NaBH_4$ for 60 min at 70°C). The proposed workflow for sequential microwave-assisted N- and O-glycomics is presented in Scheme 5.1.



Scheme 5.1: Proposed workflow for sequential microwave-assisted N- and O-release

The proposed workflow is as follows: first, the glycoprotein of interest will be digested by microwave-assisted trypsin digestion, the sample will be applied to a boronic acid affinity column, separating the N- and O-glycopeptides from the unglycosylated tryptic peptides. A peptide mass fingerprint of the unglycosylated peptides will then be obtained by MALDI MS and the protein may be identified by this data. The fraction containing the N-and O-glycopeptides will be de-N-glycosylated by microwave-assisted PNGase F enzymatic release. The PNGase F reaction may also be performed in ^{18}O water in order to facilitate glycosylation site determination by the addition of a label into the formed aspartic acid. The peptides and N-glycans will be separated by SPE C18 cartridge. ESI MS^n of the fraction containing the former N-glycopepitdes and O-glycopeptides will allow N-glycosylation site determination, structural analysis of the released N-glycans will also be accomplished by ESI MS^n.

The fraction containing O-glycopeptides will then be split in half, one half of the sample will be de-O-glycosylated by the microwave-assisted DMA/ $DMAd_6$ method for glycosylation site determination. Finally, the other half of the sample will be de-glycosylated by the microwave-assisted reductive release method and the detailed O-glycan structure may be determined by ESI MS. The boronic acid affinity columns are currently being evaluated and were not applied in this work. Herein, the first attempt at sequential microwave-assisted N- and O-release is presented.

Experimental Methods

Materials

PNGase F (glycerol free) kit was purchased from New England Biolabs (Ipswich, MA) All other materials utilized in this chapter were described in Chapter II: Experimental Methods.

Microwave-Assisted Protein Digestion

Bovine fetuin (1mg) was dissolved in 200 µL 8 M urea/ 0.4 M ammonium bicarbonate and reduced with 20 µL of a freshly prepared 45 mM DTT solution. The mixture was microwaved for 15 min at 50°C. The solution was cooled to room temperature before alkylation by addition of 10 µL of a freshly prepared 100 mM IAA solution at room temperature in the dark for 15 min. The reduced and alkylated protein sample was diluted with 690 µL water then digested in the microwave reactor with trypsin 1/20 (wt/wt) for one hour at 37°C. The reaction was terminated by addition of several drops of TFA, then the samples were dried in a speed vac. The peptides were further purified using a C18 SPE column as described in Chapter II: Experimental Methods

Microwave-Assisted de-N-glycosylation

The tryptic peptides of bovine fetuin were de-N-glycosylated utilizing PNGase F. The tryptic peptides were dissolved in 45 µL water and 1 µL PNGase F. The solution was microwaved at 50°C for one hour. Once the reaction was complete, the solvents were removed by evaporation in a SpeedVac, the samples were then further purified.

de-N-glycosylated Sample Clean Up by C18 and PGC columns

De-N-glycosylated samples were dissolved in 1 mL of a 2% aqueous ACN/ 0.1% TFA solution then applied to a C18 column equilibrated with the same solvent. Unretained N-glycans were collected and the column was washed with 6 mL 2% aqueous ACN/ 0.1% TFA. The de-N-glycosylated peptides were eluted with 6 mL 75% aqueous ACN/ 0.1% TFA and dried in a SpeedVac, the MALDI MS peptide mass fingerprint was then obtained as described below. The fraction containing the N-glycans was dried in a SpeedVac then dissolved in 1 mL water and applied to a hand packed PGC column which had been equilibrated with water as described in Chapter II: Experimental Methods. Following purification, N-glycans were analyzed by negative mode ESI MS.

Microwave-Assisted O-glycan release

The de-N-glycosylated peptides were dissolved in 50 µl water. From this sample, 25 µl was added to a 10 mL Pyrex reaction vessel along with 500 µL of a 40% aqueous DMA / 1M $NaBH_4$ solution. A small stir bar was added to equalize heating throughout the reaction. The microwave reaction temperature was set at 70°C for one hour. The reaction was terminated by the addition of 1.5 mL acetic acid in an ice bath. Solvents were removed by evaporation in a SpeedVac. Borates were removed by repeat evaporations with 1% acetic acid in methanol under a stream of nitrogen gas.

O-glycan Sample Clean Up and Permethylation

The O-glycans were purified by PGC column then permethylated as described in Chapter II: Experimental Methods. Following permethylation, the O-glycans were analyzed by ESI MS

MS Methods:

Electrospray ionization mass spectrometry (ESI MS) and Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) were performed as described in Chapter II: Experimental Methods. ESI MS was performed in both positive (for O-glycans) and negative (for N-glycans) ion mode.

Results and Discussions

To demonstrate how the microwave-assisted de-O-glycosylation methods developed in the previous chapters fit into overall microwave-assisted glycomics, a sample of bovine fetuin was first reduced, alkylated and digested with trypsin in the microwave reactor. The peptide mass fingerprint, which is shown in Table 5.1 and Figure 5.1A, represents 21% sequence coverage. This low sequence coverage can be attributed to the fact that all of the large tryptic peptides of bovine fetuin contain glycosylation sites and were not able to be efficiently detected.

A microwave-assisted PNGase F de-N-glycosylation was then performed on the tryptic peptides. All the same tryptic peptides were seen after the reaction, which confirmed that the reaction had no adverse effect on the peptides. Additionally, a new peak after de-N-glycosylation was evident which

corresponded to a former N-glycosylated peptide with a conversion of the aspargine residue (N) to aspartic acid (D), this data is shown in Figure 5.1B and Table 5.1. Of the three N-glycosylated peptides of bovine fetuin, only one was observed after de-N-glycosylation. The other two peptides are high mass peptides and were not able to be efficiently detected. Therefore, the glycopeptide enriching strategy should be performed before de-N-glycosylation to decrease the peptide field and increase detection of the former N-glycosylation site, which will be necessary for ESI MS^n analysis of the N-glycosylation sites.

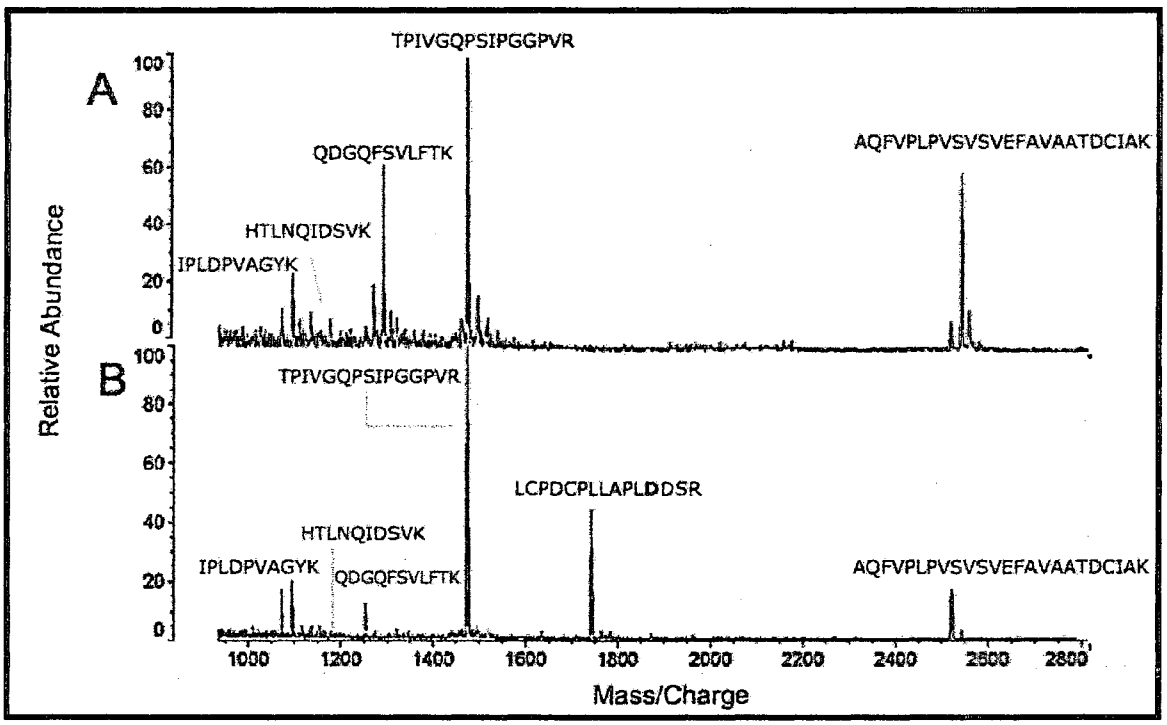


Figure 5.1: MALDI MS of the tryptic peptides of bovine fetuin (A) before and (B) after microwave-assisted de-N-glycosylation

Table 5.1: Tryptic peptides of bovine fetuin observed before and after microwave-assisted de-N-glycosylation. Known glycosylation sites are shown in bold (X=Peptide was observed in the spectrum)

Tryptic Peptide Sequence	$[M+Na]^+$	Before de N-glycosylation	After de-N-glycosylation
VTCTLFQTQPVIPQP QPDGAEAEAP**S**AVP DAAGP**T**P**S**AAGPPV A**S**VVVGPSVVAVPL PLH R	6036.13		
RPTGEVYDIEIDTLE TTCHVLDPTPLA**N**C SVR	3692.76		
VVHAVEVALATFNAE S**N**GSYLQLVEISR	3037.57		
AQFVPLPVSVSVEF AVAATDCIAK	2540.31	X	X
EPACDDPDTEQAAL AAVDYINK	2427.07		
HTFSGVASVESSSG EAFHVGK	2141.00		
QQTQHAVEGDCDIH VLK	1998.94		
LCPDCPLLAPL**N**DS R	1761.83		X (N-->D)
TPIVGQPSIPGGPVR	1495.83	X	X
CDSSPDSAEDVR	1358.52		
QDGQFSVLFTK	1290.64	X	X
HTLNQIDSVK	1175.60	X	X
IPLDPVAGYK	1093.60	X	X

Figure 5.2 shows a negative mode ESI MS of the N-glycans released from bovine fetuin after microwave-assisted enzymatic de-N-glycosylation. Each of the four known N-glycans (shown as cartoons labeled N1-N4) were detected.

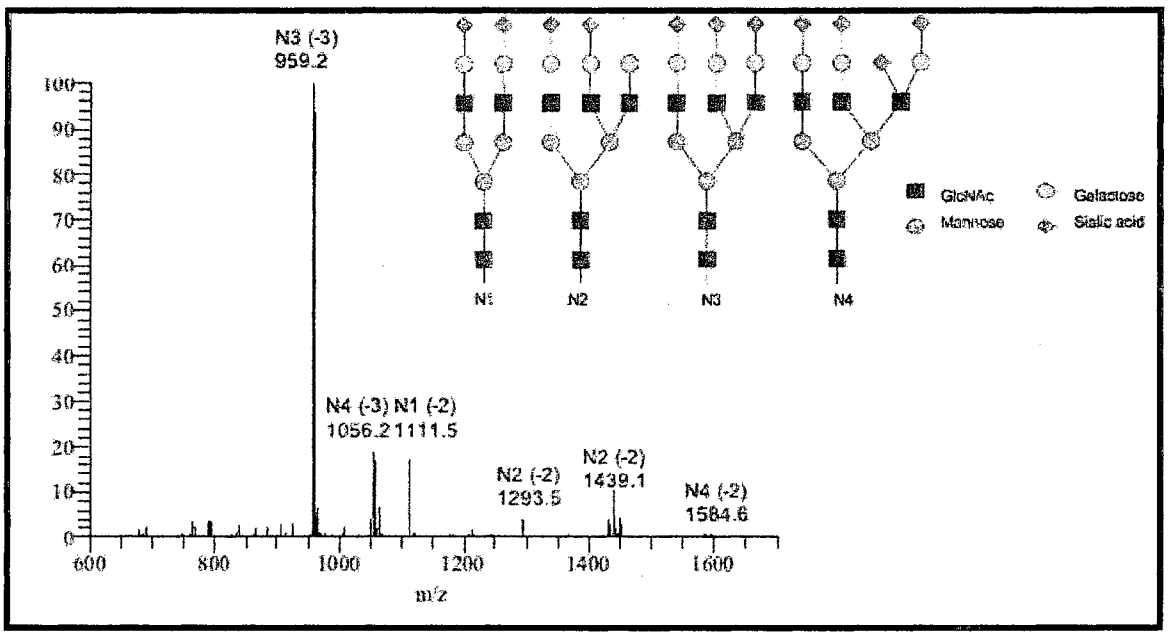


Figure 5.2: (-) ESI MS of the N-glycans released from bovine fetuin by microwave-assisted PNGase F release.

After de-N-glycosylation and separation of the peptides from the N-glycans by SPE, half of the recovered peptides were subject to microwave-assisted reductive O-glycan release and the ESI MS of the obtained O-glycans is shown in Figure 5.3, both O-1 (H1N1A1) and O-2 (H1N1A2) O-glycans were observed.

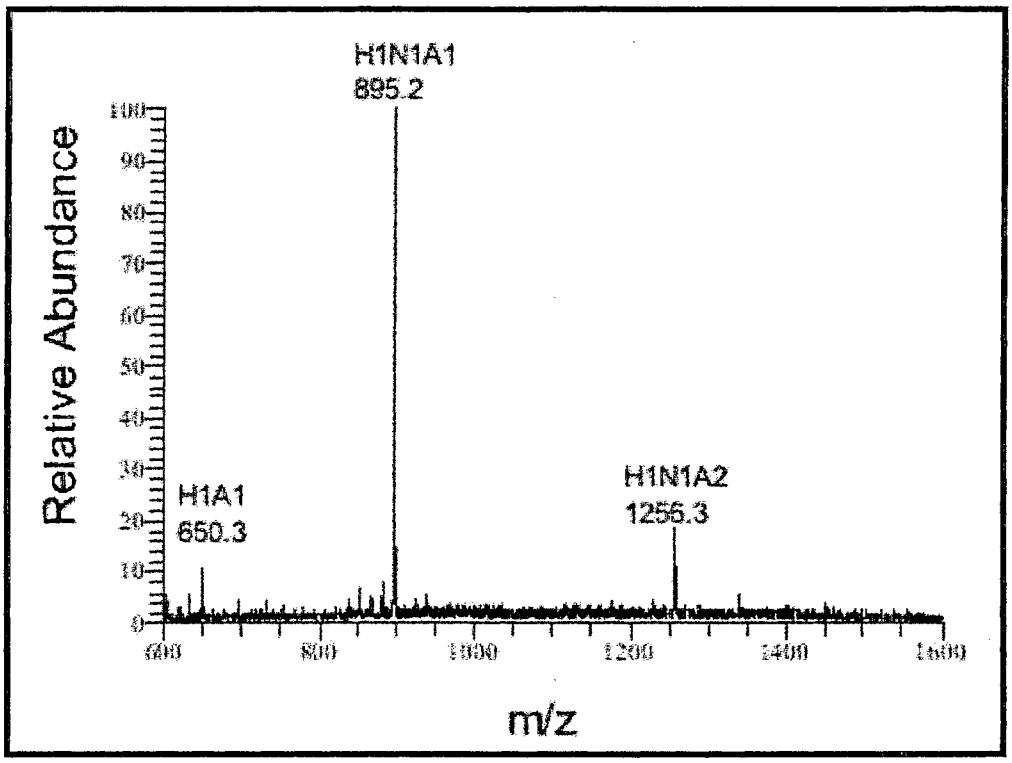


Figure 5.3: O-glycans released by microwave-assisted reductive de-O-glycosylation from a sample of bovine fetuin following microwave-assisted tryptic digest and de-N-glycosylation

Unfortunately, in the case of bovine fetuin, all four O-glycosylation sites are on one tryptic peptide. This O-glycopeptide was not able to be detected before or after the de-O-glycosylation reaction. It is again suggest that glycopeptide enriching strategies, such as boronic acid affinity chromatography, need to be applied, then DMA based de-O-glycosylation, as outlined in Chapter III, would be performed and the glycosylation site would be determined. Importantly, all results presented in this chapter were from the same 1 mg starting material sample of bovine fetuin.

Summary

The microwave-assisted methods developed in Chapters III and IV can easily fit into an overall microwave-assisted method for sequential N and O-glycan analysis. However, for use in our laboratory, glycopeptide enriching strategies must be employed, which were not available at the time of this study. These methodS, coupled with capabilities for microwave multiplexing, such as sample holders for up to 20-samples at a time, will be a powerful method for high throughput O-glycomics.

CHAPTER VI

CONCLUSIONS AND FUTURE WORK

Three novel microwave based methods for release of O-glycans were developed. The methods are summarized in Table 6.1. The methods utilized the benefits of microwave radiation to decrease reaction time, minimize unfavorable side reactions, and/or increase product yield over conductive heating-based protocols. Two of these methods; microwave-assisted reductive O-glycan release and microwave-assisted de-O-glycosylation for site determination fit into an overall scheme for sequential N- and O-release and glycosylation site determination.

Table 6.1: Summary of de-O-glycosylation methods developed in this thesis

Microwave-assisted Method	Sample prep	Microwave cond.	Release yield	Drawbacks	Advantages
Non reductive O-glycan release	aqueous DMA satd. w/ ammonium carbonate	70℃/60min	<< Classical Method	-Poor Release efficiency due to presence of ammonium carbonate. -Significant degradative peeling.	Non reduced glycans may be quickly obtained for reductive amination prior to HPLC analysis.
Reductive O-glycan release	aqueous DMA /1M $NaBH_4$	70℃/60min	> Classical Method	Peptide information is lost.	Reduced glycans are obtained in higher yields and shorter reaction times than classical methods.
De-O-glycosylation for site determination	aqueous DMA	60℃/30min	~75%	-Complete release is not achieved. -Asn-Pro hydrolysis	-DMA labeling of glycosylation site. -Addition of DMA/ $DMAd_6$ mixture allows for selection of glycopeptides.

The microwave-assisted non-reductive O-glycan release method was shown to release O-glycans with free reducing ends which then were further derivatized by reductive amination. Also, no adverse effects on peptides were observed. However, the release efficiency compared to classical methods was low. If this project were to continue, further work would involve extending the reaction time and varying temperature to accurately evaluate the optimal reaction conditions for maximum de-O-glycosylation. Additionally, it would be advantageous to be able to label the glycans *in situ* as they are released, this way less degradative peeling would occur, and release efficiency would not be hindered by the addition of ammonium carbonate. Unfortunately, conventional reductive amination relies on acidic conditions, as the best reaction yields have been determined with acid catalysts that have a pKa of about 2,[60] further research into this area would be necessary if this project were to continue.

The microwave-assisted reductive O-glycan release strategy is a fast, highly sensitive method which consistently provided higher yields of O-glycans than classical methods. Even with extreme variations in the classical method, O-glycan yields with the microwave-assisted method surpassed classical heating in all situations. Further evaluation of classical heating methods, as well as

utilization of this method for O-glycan determination on glycoproteins of biological interest would be the next logical step.

Microwave-assisted de-O-glycosylation for glycosylation site determination proved that 40% aqueous DMA with microwave conditions of 70°C, one hour released O-glycans with an efficiency of 95%. A method for 75% release efficiency, of 60°C, 30 min microwave heating for glycosylation site determination was then determined as ideal. As a next step, the peptide breakdown encountered with the harsher reaction conditions (1 hr, 70°C) should be further evaluated with standard peptides to determine if any rules exist as to which amino acids are prone to cleavage under these conditions, since they provided more complete release. Also, work on glycopeptide enriching strategies, specifically boronic acid affinity chromatography, to isolate O-glycopeptides from a mixture of peptides prior to de-O-glycosylation for glycosylation site determination is the final necessary step towards complete N- and O- glycan and glycosylation site analysis from a single sample.

Finally, utilizing the methods put forth in this thesis towards overall microwave-assisted sequential N- and O-glycan release, as outlined in Chapter V, will allow for complete, high-throughput, accurate glycomics.

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